

nature

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A hundred million dollars' worth of holes

LAST year, at the instigation of Dr Eugene Shoemaker of the California Institute of Technology, about 50 United States geologists, geophysicists and representatives of the drilling industry put their heads together to talk about deep drilling in the Earth's crust for scientific purposes. Not surprisingly, they came out in favour of it. Their proposal, aimed at anyone who will listen, is for a large scale continental drilling programme within the United States and is just published*. The recommendations are sufficiently wide-ranging and expensive (indeed they have rather a 1960s air to them) to merit a long critical look by the scientific community and some very careful thinking in Washington.

The primary purpose of the Continental Drilling Project (CDP), according to the report, is "to provide a sound scientific base that will aid government in establishing national energy policy and that will assist industry in the discovery and development of new energy policies". Then, a page later, the "objective of the proposed drilling program is not for mineral exploration itself but for scientific data ... helpful to geoscientists in developing sound theories on mineral deposit locations". But then a quarter of the funds are to go to investigations of the nature of faulting and earthquakes—the public safety line. All this in a frantic ten pages of foreword, preface, summary and recommendations and introduction. Clearly the net is being cast wide for potential sponsors.

Holes from 1 to 10 km deep could, it is claimed, make major contributions in at least the following fields:

- Lateral penetration of a fault zone to study near-fault conditions, even with the idea of thinking seriously of earthquake control.
- Drilling into hydrothermal systems and active magma chambers in an attempt to understand their physics and geology.
- Determination of the thermal structure of the Earth's crust.
- Investigation of the state of ambient stress in the crust.
- General studies on the deep geology of the United States.

In addition, a programme of shallow drilling (down to 300 m) is proposed in which three teams sweep over the United States, drilling 500 holes a year for a variety of purposes. This last programme, at \$1 million a year, looks sufficiently sensible (and certainly necessary) to be implemented independently of the success or failure of the rest of the proposals.

What of the remainder—can it be done, should it be done and if so, by whom?

The report makes it clear that drilling to a depth of 10 km in crystalline rock will be pushing technology quite hard. Holes to that depth exist in sedimentary formations (in pursuit of oil), cost up to \$10 million and take up to

700 days to drill. In hard rock the costs would be comparable and the time required somewhat greater, but costs and times would obviously increase if extensive scientific investigations were carried out simultaneously. The instrumentation requirements for such work demand considerable improvements in temperature protection over present-day equipment. But there do not seem, as far as the technology goes, to be any fundamental obstacles.

Should it be done? The past twenty years have seen some chequered fortunes in major earth-science ventures. (The name of Mohole inevitably springs to mind, but so also does the Joides programme and its successor, the Deep Sea Drilling Project. The report makes only very passing mention of the latter, and none at all, of course, of Mohole.) What emerges clearly is that not all the proposals discussed have equal merit and that it would perhaps be a mistake to try to sell a \$100 million package on the basis that everything fits together. For instance, drilling laterally into the San Andreas Fault might be worth the investment of \$10 million or \$20 million (but it is surely imprudent to talk yet of control), whereas many of the less specific projects aimed at generalities like understanding the underlying structure of the North American continent barely warrant the same attention. It is particularly unfortunate that the whole is tied together with vague claims about energy policy and mineral resources. The veneer which it is necessary to apply to proposals these days is often distinctly thin, if not transparent. A wise decision in Washington would be to be very selective of the pack and to ignore the packaging.

And if support is forthcoming, who will get it? Drilling companies will, of course, and will handily find the government supporting their research and development. On the scientific management front, the division has somehow to be worked out between government institutions, particularly the Geological Survey, and the universities. Giving the lion's share to the Geological Survey will cause a lot of bitterness in the academic world among those who think the survey writes its own cheques, but at least the job will get done. What the survey fears, no doubt, is that it will be entrusted with day-to-day running, with the universities coming in when things get exciting and creaming off all the interesting material.

Finally it is regrettable that in the earth sciences, traditionally most internationally minded, it is now desirable to write a report which barely touches on international collaboration. A paragraph is devoted to mention of collaboration in Iceland. We are told in two lines that the idea (of deep drilling) has been implemented in Canada, West Germany, Japan, South Africa and the Soviet Union. And that is all. If this is a consequence of a growing nationalism in matters of energy and mineral resources, then it is a bad one, and it is to be hoped that any scheme ultimately approved takes note of the international dimension. □

*Continental Drilling (ed. by Shoemaker, E. M., Carnegie Institution of Washington, 1975).



WHEN the first council of the Netherlands Organisation for Pure Scientific Research (ZWO) was ceremoniously installed on May 31, 1950, the organisation had already worked for a couple of years, and had thus gained considerable experience. Ever since, it has been held in general esteem, even by those who have not been entirely happy with what the ZWO did. Even they will readily admit that the ZWO did a great deal and that it more or less shaped the face of Dutch science. This year, a quarter of a century later, the ZWO installed its fifth council—during an equally solemn session, attended by Queen Juliana, members of the government and hundreds of prominent scientists—but it was generally felt that this might well be the last one.

As official proposals now stand, the ZWO is to merge with several other institutions to form the Council for Scientific Research (RWO). As far as Dr J. H. Bannier, the present ZWO director, is concerned, this would be just fine, with some important provisos.

- The RWO should have its own independent funds, with which it could react quickly to any request for support, without any of the red tape involved in government ministries.

- The RWO should be able to manage research (mainly) by progress control.

- RWO should have far better formal contracts with universities than is provided for in the government proposals. (The ZWO has two representatives for each university and one for each specialised school, such as the institutes of technology, on its council, and even that is not found quite sufficient.)

- The RWO should provide inter-disciplinary contacts.

- The RWO should not manage more than 20% of the total budget for fundamental research, albeit in co-ordination with the other, equally independent channel for money supply—the government through direct support for universities and other institutes.

It is difficult to determine just when the idea of an organisation for pure scientific research was born. Some trace it back to the German concentration camp of St Michielsgestel in the Netherlands, where a considerable number of prominent Dutchmen were kept in (relatively decent) custody—partly as hostages, partly to keep them from organising anything subversive.

This camp was a breeding ground for ideas on how to change the country at the end of the war. But when the concentration camp was liberated, the idealistic inmates found the country still at war and themselves without the means to implement what they had planned in such gloomy conditions. When at last the war was over, it took some time to find out in

how much of a shambles the country was, and even more time to realise the size of the gap between the Netherlands and countries that had not been occupied.

Once this had been established, however, things proceeded at a fantastic pace. Four months after the war, a new, more or less provisional government took over power from the military, and scientists started to meet again. They decided that a prominent member of their community should go to the USA and find out what the state of science was and how that country had managed such scientific marvels

ZWO: past present and future

Arie de Kool reports from Rotterdam on the evolution of the Netherlands Organisation for Pure Scientific Research, which is 25 years old.

as the atom bomb. This scouting job was assigned to Professor Vening Meinesz, a geophysicist.

Physics seemed the most promising field, together with mathematics (the first computers had been built). A month after Meinesz returned, in January 1946, a mathematical centre was founded in Amsterdam, and in March 1946 the Minister of Education and Science, Van der Leeuw, one of those detained in St Michielsgestel, was instrumental in establishing a Foundation for Fundamental Research on Matter (FOM).

This foundation was immediately successful, and has been ever since. It has become the model for virtually all ideas in the Netherlands on how to organise science. Even the fiercest critics of the organisation still maintain the basic model when they propose their alternatives.

The main instrument is the 'working community'—in which each particular speciality is discussed. So there is a working community for solid state physics, one for nuclear physics, one for high energy physics, one for plasma physics and so on. The working communities submit their plans to the FOM and the council then decides who gets what, basically on scientific merit only; in practice, however, scientific, technological and social merits cannot always be clearly separated.

By August 1946 a report was brought out proposing the establishment of an overall organisation for scientific research, working essentially along the same lines as the FOM but on a larger scale.

There had been elections in the meantime, however, and a new government had come to power; the new Minister of Education and Science seemed to be far too busy restoring the ministry to its pre-war status to have either time or interest to devote to science and its organisation.

It took about 6 months to get any reaction at all, and he finally conceded that if those scientists absolutely wanted to, he would not keep them from continuing their study on how to organise science.

The scientists went further than that. They set up a provisional organisation for the stimulation of science by judgment (and selective support) of proposals. The organisation had one foot in the ministry itself: its secretary (a gift from the former minister) was a public servant who had had a scientific education and who headed the office of higher education and science—J. H. Bannier.

After a year the first chairman of the committee (director of the organisation) accepted a professorship and left, and by that time it had become a matter of course that Bannier would take his place. It took two more years, however, for the government to pass a law to establish the ZWO officially. During the first four years it worked merely as an advisory body, for the government did not really feel like handing out large amounts of money to scientists to divide among themselves. This meant that for every penny earmarked for the support of some project, at least 25 signatures from different departments were needed before the money could actually be made available.

In the first year the ZWO received the full amount of Dfl 1.5 million (\$400,000 or £130,000 at that time). More than half of this was earmarked for the FOM which, with some other foundations, was made a sort of independent subsidiary of the ZWO. Last year, the ZWO administered some Dfl 125 million, the equivalent of \$50 million or £20 million. Dutch government expenditure on research and development totals some Dfl 2,000 million (\$800 million or £350 million), while the total research and development expenditure of the Netherlands is about twice that amount. Universities are thought to spend some Dfl 1,000 million on research, but nobody is very certain about that.

In practice, the ZWO's influence is much greater than it might seem simply from these figures. In physics there is hardly any research done outside the coordinating influence of one of the working communities of the ZWO's 'subsidiary', the FOM. This is not quite the case in other disciplines,

although the ZWO subsidiary FUNGO (Foundation for Fundamental Medical Research) claims that it really controls about twenty times the amount of research it pays for. Other ZWO foundations (for pure chemistry, biological research, biophysics, and some smaller areas) claim a similar, but smaller, influence.

The method followed is quite simple. The ZWO (or one of its foundations) may support a project by providing one extra man, or a piece of costly machinery, or just the costs of publication. In the total project budget this may be a rather minor point, and a condition is, of course, that the project be brought in for assessment and co-ordination.

Lately, however, there has been

some criticism of this method. Not on scientific grounds, for almost everybody agrees that the system is working well (although some would want more openness and some would go for a more democratic procedure in appointing the judges). But the method implies the existence of temporary positions—appointments of scientists for the duration of just one project.

Temporary positions were generally accepted for as long as scientific posts were plentiful. Doing a project for the ZWO (often as a way of obtaining a PhD) could often help a person to find a more permanent position. Now that the market is getting really difficult, people are wondering if temporary appointments are still socially acceptable. The ZWO does not want to

behave in a socially irresponsible way, and the organisation has set out to ensure that its employees receive a normal income when unemployed after a temporary appointment. But according to Professor R. van Lieshout, now managing director of the ZWO and professor of physics at Amsterdam, the problem has not yet had its full impact. Unemployment is most serious where it has always been a problem—among social scientists mainly—and physicists can still find employment. But Professor van Lieshout does not have many words of comfort for ZWO scientists generally. His message is: "I advise most people on a temporary contract with the ZWO to find themselves a permanent job as soon as possible." □

international news

WITHIN 10 extraordinary days, the mood of science in the political fabric of Australia has passed from one of constructive optimism to destructive despair. The change has mirrored an upheaval within the Labour government precipitated by a massive reshuffle of Cabinet portfolios by Prime Minister Gough Whitlam announced late on the night of June 5. Australian scientists have been shocked to find their interests and their principal research organisation, CSIRO, subject to a totally unexpected battering. They have a new minister, Mr Clyde Cameron, who came into the portfolio with a public scream, and CSIRO is subject to surgery without the courtesy of consultation.

On May 29, only seven days before the Cabinet reshuffle, the long awaited Australian Science and Technology Council (ASTEC) held its inaugural meeting in Canberra. The formation and role of ASTEC had been announced by Mr Whitlam at the big ANZAAS Congress in Canberra in January but virtually nothing of significance followed the ASTEC announcement until late May. The then Minister for Science, Mr Bill Morrison, had expected ASTEC to get underway rapidly after the ANZAAS announcement, but as seemed to happen with many things in his 2½ years of scientific jurisdiction, matters ground along slowly.

It took four months to settle the interim membership of ASTEC ("interim" because it has been established before the passage of legislation to give it statutory independence). Rumours

Australian science in turmoil

from Peter Pockley, Sydney

had flown around the scientific community of the difficulty Mr Morrison was said to be having in getting the agreement of people to serve. Probably more to the point was Mr Morrison's lack of regard for many of the longer standing leading figures of Australian science, who, he was heard often to say, kept cropping up repeatedly on government committees. As a result he was not disposed to appoint to ASTEC many of the names put up, for instance, by the Australian Academy of Science.

The final 12 nominations, although lacking weight in some fields, did show some ingenuity in the balance of interests represented, thereby lending credence to Mr Morrison's intention that ASTEC should have an influence on the wide range of government policies on which science and technology have a direct, if hitherto unrecognised, impact. The crucial post of chairman went to Dr J. A. Louis Matheson, an engineer by background, who will retire as Vice-Chancellor of Monash University at the end of the year, when he will be able to take up his ASTEC duties on a full-time basis.

The other part-time members can be

classified in various ways—six university professors, two technologists, a managing director of a technological firm, and a trade unionist. The appointment of a philosopher, Professor John A. Passmore of the Australian National University, was an imaginative move (he had taught Mr Morrison years earlier) and the inclusion of Professor Sol Encel, a sociologist at the University of New South Wales and one of Australia's few students of science policy as a discipline, has been an insurance against ignorant parochialism.

The Australian National University, established in the national capital, Canberra, has four members on the council. The leading medical researcher and administrator, Professor Gustav J. V. Nossal, Director of the Walter and Eliza Hall Institute of Medical Research in Melbourne, will be sure to keep ASTEC interested in the strategy of Australian medical research, an area which earlier had been thought likely to be specifically excluded from ASTEC's purview.

The total number of members announced in mid-May was 11, not the 12, and this shortfall of one brought out the first sign of political trouble ahead for science in Australia. In announcing his 'First Eleven', Mr Morrison told the world that the Premier of the State of New South Wales, Mr Tom Lewis, had refused to make available the services of Mrs Marlene Brell, a teacher, nutritionist, and (above all, for Mr Morrison who espouses both the consumer and female causes) a woman and a

prominent consumer advocate. Mrs Brell had the misfortune to be employed under a state authority, and just now there is a spate of bitterness between the Liberal-Country Party government of New South Wales and the Labour government in Canberra. A futile slanging match followed between the politicians, and ASTEC still has only 11 members.

In a constructive move to keep science away from party politics in Canberra at least (a wise, if rare, action in the light of the fractious mood of the national Parliament where the Opposition uses its majority in the Senate to defeat much of the government's legislative programme), Mr Morrison invited his opposite number in the Liberal Party to the first ASTEC meeting. Mr Eric Robinson had succeeded Mr Tony Street as Opposition spokesman on science two months earlier, following a leadership struggle in the Liberal Party which saw Mr Malcolm Fraser, a former Minister for Education and Science, emerge as the new Leader of the Opposition. Mr Street had drafted the Liberal Party's first statement on science policy in March (Liberals, including Mr Fraser as minister, had previously denied con-

sistently that a policy for science was even capable of definition); this differed little in detail from the Labour platform.

The omens for a bipartisan approach to science were good. And necessary, too, for Mr Morrison's lack of real conviction that science needed increased support had resulted in a slow but serious downward drift in public support for research and development compared with a massive injection of government funds into other areas such as social services, health services, education and urban development. Mr Morrison's belief that there are no votes in science had led him to look elsewhere for political kudos; he found some by grafting the government's nascent interest in consumer affairs on to his portfolio, and he found some more by maintaining his vestigial responsibilities under Foreign Affairs for the nearly independent nation of Papua New Guinea and an assistant role in Defence.

With ASTEC one week old, Mr Morrison achieved his ambition for promotion out of the lowly Science portfolio to Defence. The reshuffle which allowed his move came about through the resignation of the Minister

for Defence, Mr Lance Barnard, to take a diplomatic post.

Mr Whitlam and his government, which scraped back with a majority of five in an election 12 months ago, were plagued with inept performances by several of the over-large 27-man Cabinet. Mr Barnard's resignation gave him the chance to promote the able ministers and demote or transfer to supposedly harmless duties the incapable and the stubborn. One in the last category was Mr Clyde Cameron, a determined and capable Minister for Labour and Immigration, who nevertheless fell foul of Mr Whitlam through his intransigent support of inflationary wage demands. One by one, the Prime Minister placed his ministers in new slots (he has no choice over the people in the ministry who are elected by the whole Labour Caucus); the last slot left was Science and Consumer Affairs (a new portmanteau title, thanks to the outgoing Minister, Mr Morrison) and the last minister he contacted was Mr Cameron. His old portfolio had already been allocated to another, and Mr Cameron refused to serve. He held out for the best part of the day while the other ministers were sworn in; the Prime Minister began formal arrangements with the Governor-General to have Mr Cameron dismissed. The reluctant and unsmiling Mr Cameron eventually caved in and accepted. No Australian minister responsible for science had achieved such dramatic publicity. And Australian science has never received such unwarranted notoriety.

● Next, CSIRO was thrown into turmoil. Radical action of a kind previously uncharacteristic of Australian scientists, and CSIRO ones in particular, has taken over from their normally very private and cautious approach to political matters. On June 13 a series of unprecedented stop-work meetings were held by CSIRO staff around Australia.

The precipitating cause of the trouble was a sentence at the very end of the Prime Minister's press statement issued late on June 5 in which the new ministry was announced. At that time the Cameron controversy was still unresolved, and because several other senior ministers had their wings clipped to fit more junior posts, the Canberra press had time neither to read nor report the couple of pages at the back of the press statement which announced "The Department of Minerals and Energy will take over responsibility for the Minerals Research Laboratories and Solar Energy Studies Unit".

Nobody in the press gallery noticed that this meant a carve-up of CSIRO under which these laboratories and unit were developed from scratch. What is more extraordinary is that the Chair-

IN a move which caught space scientists and NASA officials by surprise, the House Appropriations Committee last week voted to decimate the budget for the Venus Pioneer programme, which is one of NASA's priority planetary missions for the late 1970s. The committee has left only enough money in the budget to "maintain a management capability", and recommended that work on Venus Pioneer be deferred for a year while the mission's priority is re-examined. Specifically, the Committee suggested that a choice may have to be made between the Venus mission and the Large Space Telescope (LST), one of NASA's major astronomy programmes.

Before the committee's recommendation is put into effect, it must be approved by both the House and the Senate. The House is likely to follow suit in view of the empty rhetoric being voiced in Congress about the need to reduce federal spending, but NASA officials are trying to persuade the Senate Appropriations Committee to leave the mission intact. At this stage, it is uncertain whether they will succeed, but the immediate outlook for the Venus Pioneer programme should be considered dim.

Scheduled for launch in 1978, the Venus Pioneer mission would consist of two spacecraft, the first of which would study the planet from a highly

elliptical orbit while the second would send a number of probes into the atmosphere. It would build extensively on the Mariner 10 results.

The House Appropriations Committee suggested that the mission should be deferred (the next launch window would be 19 months later) "to permit a budget priority decision in 1977 between the Large Space Telescope and Pioneer Venus". The committee made that recommendation because "some astronomers have been critical of NASA's Space Science programme because they contend that a disproportionate level of NASA dollars have been used on planetary astronomy missions, while little or no funds have been allocated to deep space astronomy which is the mission of the Large Space Telescope".

The LST, which was accorded top priority in a report issued earlier this year by the Space Science Board of the National Academy of Sciences, would be a large optical telescope placed in Earth orbit by the space shuttle early in the 1980s. Although some questions have been raised about its priority, debate has mostly been on the merits of the LST compared with a mission to Jupiter and Uranus. To pit the LST against the Pioneer Venus mission, a NASA official remarked last week, "opens up a whole new ball game".

Colin Norman, Washington

man, Executive and staff of CSIRO did not learn about their loss (let alone have any forewarning of the announcement) until the next day, June 6, a day on which their reluctant new minister was totally involved with his own fate.

The Minister for Minerals and Energy is Mr Rex Connor, who with his Departmental Head, Sir Lennox Hewitt, is tough, intractable, inaccessible and uncommunicative. The main scientific bodies under the Minerals and Energy portfolio are the Australian Atomic Energy Commission (AAEC) and the Bureau of Mineral Resources, both worthy but lacking any scientific high-fliers. Since coming to office, Connor and Hewitt have brooked no opposition in building their empire of bureaucracy, legislation and influence; lately they have been helped by their highly political scientific adviser, Professor Harry Messel, no mean empire builder himself as Head of the School of Physics at the University of Sydney. Messel was recently appointed a part-time Commissioner on the Atomic Energy Commission and is being tipped as the next Chairman. Connor has had ambitions to turn the AAEC into an energy research body not specialising in nuclear power, but lately he has appeared almost obsessed with the prospects of Australia developing a massive, indigenous industry for uranium enrichment—under his control, of course—and there have been broad hints, with no specific evidence in support, that Australia has developed its own enrichment technology.

The tight-fisted control and the secretive style of the Minerals and Energy operations, at both the political and bureaucratic levels, have been privately deplored by CSIRO and academic scientists. Yet, for over two years CSIRO has officially been trying to make effective contact with Minerals and Energy for collaborative projects on energy research; most of their efforts even to establish contact have been ignored.

These facts have compounded the shock felt throughout CSIRO by the astoundingly successful raid on their domain carried out at a time of such political confusion. The raid had been planned over many months—at least since the appearance of the OECD report in mid-1974 on Australian science policy which had criticised Australia for having no effective energy policy, a statement to which Connor took furious exception. The gentlemanly scientists of CSIRO simply had erected no defensive barriers to deter the raiders from implementing their plot. The Chairman of the CSIRO, Dr Jerry Price, has effectively put his job on the line by calling a press conference in which he roundly

condemned the government's move. He has the support, though, of the CSIRO Advisory Council, a body of eminent outside experts, who in their first-ever emergency meeting unanimously criticised the Prime Minister and are seeking an audience with him "to propose alternative ways in which the objectives of the government in regard to minerals and energy could be better achieved than the present proposal". Dr Price said the carve-up of CSIRO could mark the beginning of the end of the 7,000-strong organisation as an integrated operation and also as one with a high level of purposeful morale.

The staff of CSIRO have become politically active as never before. Petitions, telegrams and representations to politicians, mass meetings, a one-day strike (done by the rather quaint method of staff applying *en masse* for a day's leave, with the concurrence of their chiefs) and press publicity have taken over from research for the moment. The Liberal Opposition has rediscovered science as an issue to use against the government. The Chairman of ASTEC, Dr Metheson, his council barely functioning, has protested at being bypassed; the questions involved in the organisation of energy research should have been examined by ASTEC if this innovatory organisation is to have any meaning or influence. □

More armament and no disarmament

from a correspondent

THE Stockholm International Peace Research Institute (SIPRI) published its Yearbook for 1975 last week. As usual, the 600-page volume catalogues the last twelve months' developments in world armaments and disarmament. As usual, it makes sobering reading.

The general picture emerging from the Yearbook is one of a world approaching the threshold of widespread proliferation of nuclear weapons. The Indian nuclear explosion in May 1974 was a dramatic demonstration of the ease and cheapness with which a country with a significant peaceful nuclear programme can produce nuclear explosives. (The explosion probably cost no more than \$400,000, including the cost of the plutonium and the preparation of the test site.) At the end of 1974, 19 countries had operational nuclear power plants producing a total of about 73,000 MWe; and if present plans are carried out there will, in 1980, be 28 countries with nuclear power reactors capable of generating a total of about 300,000 MWe. Looking further ahead, by the year 2000 it is probable that the 1980

figure will have multiplied more than tenfold. The Yearbook draws attention to the amount of plutonium that these reactors will produce: 350,000 kg in accumulated stocks by 1980. This situation will give rise to two sorts of danger: first, manufacture of nuclear weapons by countries with plutonium, after the Indian example, and second, nuclear terrorism.

The recent conference held in Geneva to review the Nuclear Non-Proliferation Treaty (NPT) was intended—and completely failed—to strengthen that treaty, the only political barrier to proliferation. In the light of the failure of the conference, and given the relative technological ease with which nuclear weapons have been shown to be produced from peaceful nuclear programmes, there will be little incentive for countries with plutonium stocks to refrain from producing nuclear weapons if they perceive that these weapons would strengthen their security. Nuclear terrorism will be facilitated by the very amount of plutonium existing by 1980. Even if it is safeguarded with 99.9% efficiency, the weight diverted from its intended use will be enough to manufacture a bomb a week.

The threat of nuclear proliferation tends to obscure the fact that nuclear weapons and their delivery systems account for only about 10% of the total world military expenditure. When nuclear and conventional expenditures are considered together, the trend points to other causes for alarm. In 1974 the world spent over \$210 thousand million on armaments: a sum approximately equivalent to the total income of the poorer half of mankind. For the first time, NATO and Warsaw Pact forces between them spent less than 80% of this amount. The areas whose shares have been rising over the last 15 years are the Third World in general (whose expenditure has more than doubled), the Middle East, which has recorded a fivefold rise, and China, whose share has doubled. Thus the figures show increasingly intense procurement of armaments by more countries. The arms trade is flourishing as never before. Between 1973 and 1974 alone it increased in value by 40%. In 1974, the Middle East was the recipient of more than half the total arms supplied. The Persian Gulf states received 27%.

The high cost of salaries for military personnel—a cost amounting to a third of total military expenditure—is making automated weaponry more attractive. The USA is developing a new weapon system which could provide a fourth component to the arms race, after strategic bombers, nuclear submarines and intercontinental ballistic missiles. The new system is the

long-range cruise (non-ballistic) missile (LRCM). It can be launched either from submarines or from aircraft, and has both tactical and strategic capabilities. Its distinctive feature is great accuracy of delivery: an accuracy which is also being built into the class of conventional weapons known as precision guided munitions. Such munitions now include a whole range of air-to-air, surface-to-air, air-to-surface and surface-to-surface weapons. The conventional battlefield is taking on a new look with the development of automated aids such as night vision equipment, artificial sensors which locate targets, command and control, and information-processing systems, all of which are bringing the automated battlefield nearer and nearer.

Against this background of threatening proliferation, increasing militarisation and new weapons technologies, the Yearbook cannot point to any convincing measures of arms control or disarmament. The meeting between

President Ford and General Secretary Brezhnev in Vladivostok in November 1974 set the limit of missiles with multiple independently-targeted re-entry warheads at 1,320: more than either side had deployed at the time of the accord. Presumably such a high figure was chosen so that each side could complete its planned deployments and still reap the political benefits of having arrived at an agreement. A similar situation obtained in the Threshold Test Ban Treaty, signed in July 1974 by the USA and the USSR. Each side undertook not to carry out any tests having a yield of more than 150 kilotons. Again, this limit is so high that it does not interfere with any tests intended by either side for any tactical and most strategic nuclear weapons at present deployed or even planned. The cynicism of the agreement is even more glaring given that refinements in accuracy are presently being pursued more enthusiastically than refinements in size. In fact, 1974

was a record year for the number of nuclear explosions touched off by all countries. In all, 35 explosions were carried out: 20 by the USSR, 5 by the USA, 7 by France and 1 by each of the UK, China and India. Treaties such as the Threshold Ban do nothing to limit the number of tests carried out by the signatory states, or to slow down the arms race.

SIPRI has a way of making the facts seem compelling. And yet there is more to international relations than just facts. The Yearbooks are invaluable for their collation and analysis of facts, but they do not analyse to what extent these facts influence the behaviour of decision makers. To enter this realm would admittedly be to go beyond the task SIPRI has set itself; and yet without it the Yearbook's pessimism occasionally seems too relentless. What is indisputable, however, is that the facts add up to more armament and no disarmament. And there is no change in sight. □

correspondence

In defence of MIT

SIR,—I am not often shocked by Nature, so all the greater the impact when that happens. It has just happened as the result of reading your leading article in the issue of May 22.

It is not one of your best, witness such an impenetrable sentence as: "And one thing that ought to have been learnt is that the isolation of scientific and other intellectual pursuits, particularly from bodies which claim to be representative, profits no-one and is a futile gesture in the face of strong regimes."

But this article ends with a clear and to me utterly reprehensible paragraph, saying that not all divisiveness stems from developing nations: "it should be added that some of the initiatives of the developed world seem only capable of generating bad feelings"; and then citing as its single example of such deplorable provocations that the Massachusetts Institute of Technology made acts of religious discrimination a cause for terminating a projected contract involving water desalination with Saudi Arabia. The *Nature* editorial ends: "Saudi Arabia can hardly welcome Jews with open arms; it surely behoves MIT to use restraint and not to trigger confrontation by insisting on sending them."

The relevant stipulations were in fact not written into the projected

contract, but appeared in a covering letter from Jerome Wiesner, President of MIT. This stated in effect that under special circumstances either party could withdraw from the arrangement, and that MIT would feel obliged to terminate the contract if systematic discrimination in employment under the contract were exercised on the basis of race, colour, national origin, religion or sex. Such considerations govern all MIT employment, and are now required by American law. Saudi Arabia responded by withdrawing the proposal.

I am proud of MIT, which needed no prodding from the US government to adopt such practices since they are backed strongly by its faculty and students; and I am proud of the US government for having such laws.

But what are we to make of *Nature*?

Yours faithfully,
GEORGE WALD

Harvard University

Fossil footprints

SIR,—The account of the finding of a fossilised human footprint in Turkey (*Nature*, April 17) would seem to indicate that fossil human footprints are now becoming scientifically respectable.

If this is so, readers might be interested in comparing those found in

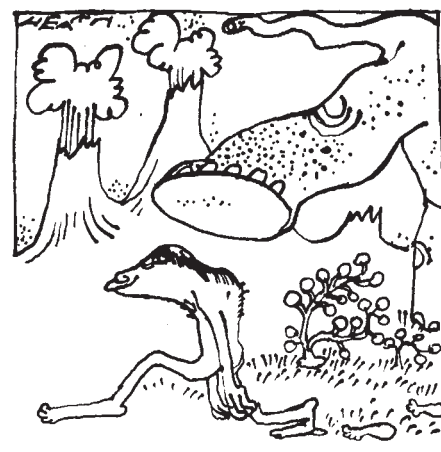
and around the Paluxy River, near Glen Rose, Texas, in rock usually identified as Cretaceous. The unusual thing there is that dinosaur footprints are found in the same strata.

These footprints have been shown in a well known film *Footprints in Stone*. Also, descriptions and comments have been given from time to time in the *Creation Research Society Quarterly*.

I have seen neither the prints themselves from Texas nor, of course, those from Turkey. On the basis of photographs, however, I can only say that the prints from Texas appear to be much better ones.

H. L. ARMSTRONG

Kingston, Ontario



news and views

Energy cost of food gathering

from Robert M. May

RECENT changes in the cost of petroleum and other fossil fuels, allied with a growing awareness that these resources are finite, have prompted many studies of the economics of food production expressed in terms of energy rather than money: how many calories does it cost to put a calorie of food on the table? In this context, it is interesting to explore the energetic book-keeping of natural animal populations.

Lawton (in a chapter in *Resources and Population*, edit. by Benjamin Cox and Peel; Academic Press, 1973) has reviewed some of the data, gathered in the field and laboratory by various people, on the number of food calories obtained for one calorie of food-gathering effort. For the natural populations surveyed, this ratio runs from around 2 to around 20, with the typical value around 10. (For natural populations, with no energy subsidies, the ratio must obviously exceed unity.) This overall similarity in the ratio of energy gained to energy expended for the various species is true despite the fact that the absolute rates of energy expenditure during foraging vary over eight orders of magnitude, from the very high-cost techniques of hovering hummingbirds (with absolute rates of energy expenditure during foraging of between 16 and 33 cal min⁻¹), to the lower values for black bass (2–3 cal min⁻¹) and bumblebees (0.3–0.5 cal min⁻¹), to the extremely low values for the relatively inactive damselfly (around 5 × 10⁻⁵ to 5 × 10⁻⁶ cal min⁻¹). These absolute differences result both from the sort of foraging behaviour, and from the different body sizes of the animals. Lawton notes that energetically expensive food-gathering techniques in animals can only be applied to food sources which guarantee a high rate of energy return, and conversely: this fact probably accounts for the observed convergence in the ratio between energy gained and energy expended, independent of whether the basic strategy is 'high cost' or 'low cost'.

In simple human societies, obtaining their food without fossil fuel energy subsidies, the ratio of food calories gained to calories invested is similar to that which pertains throughout the animal kingdom. Thus the food of the islanders on Lamotrek Atoll in the South Pacific comprises root crops, coconuts, breadfruit, and animal protein in the form of pigs, fish, turtles and sea-urchins: this is gained by a

mixture of hunting, gathering and farming, with an average ratio of energy gained to energy expended of around 20 (Odum, *Environment, Power and Society*; Wiley, 1971, after Alkire). Other data summarised by Odum and by Steinhart and Steinhart (*Science*, **184**, 307–316; 1974) suggest that hunter-gatherer societies typically average a ratio around 5–10, while shifting agriculture does better at around 20. A detailed study by Rappaport (*Scient. Am.*, **224**(8), 117–132; 1971) of the shifting cultivation practised by the Tsembaga in New Guinea gives a figure of 16. The wet rice agriculture practised for many centuries without energy subsidies in Indonesia, China, Burma and Thailand runs around a ratio of 10–50. In all, such 'primitive' cultures gain 5–50 food calories for each calorie of human energy invested, with a typical figure of around 10.

The analogous ratio in 'advanced' technological societies has been the subject of many recent analyses. The Steinharts have attempted to estimate the total number of fossil fuel calories invested, both upstream and downstream from the farm gate, in getting one calorie of food energy on to the contemporary US table. On the average, the ratio of calories output to calories input is 0.1. This figure, which differs by two orders of magnitude from that for animal and simple human populations, is generous in that it makes no allowance for private automobiles involved in getting the food home, nor for waste disposal (which currently engages 12% of all US trucks). Around the turn of the century the ratio was about 1, and it has declined steadily (averaging around 0.2 in 1940), partly in response to the growing per capita consumption of feedlot beef and other products high on the trophic ladder, and partly in response to ever more technologically intensive methods of farming. Thus even for corn, an ecologically efficient crop at the primary trophic level, current practices give an energy output/input ratio of 2.8 as the crop leaves the farm (Pimentel *et al.*, *Science*, **182**, 443–449; 1973).

In animal populations and most human societies, essentially everyone is involved in the business of getting food. (The social insects provide illuminating exceptions. But even here, the relative abundance of the various castes, and the division of labour among them, may be understood as minimising the total energy cost needed to maintain the society. The tentative beginnings of such 'ergonomic' studies are outlined by Wilson in chapter 18 of *The Insect Societies*; Harvard University Press, 1971.) In contrast, the increasing fossil fuel subsidisation of food production in the technologically advanced countries has been accompanied by significant changes in what we might call the 'ecological structure' of the work force. In the United States, farmers constituted 10% of the work force 50 years ago, and 2% today: one farmer feeds 50 people. Of course this figure is misleading, and when the people involved in all phases of the food supply system are added up they currently amount to around 20% of the work force (Pimentel *et al.*). This is still qualitatively different from essentially all previous animal (including human) societies, and leaves 80% of the work force free for less fundamental pursuits, some of which are necessary or at least useful (for example, building houses, reading *Nature*), others less so (for example, manufacturing bizarre products to gratify artificially created desires).

Many people have expounded the obvious implications of these facts in a world where fossil fuel supplies are finite and increasingly expensive: short-term problems in exporting technologically intensive agricultural methods to less developed countries; possible long-term problems in sustaining such energy subsidies in any society; artificial patterns of wasteful consumption in societies where only a relatively small proportion of the work force is deployed in truly essential tasks. But there has been very little detailed analysis of economic and social dynamics taking into account the basic ecological structure of the society. In other words, there is a lack of studies which draw a profound distinction between the demand curve for calories and protein, and the demand curve for plastic gee-gaws. Some light on the economic instabilities currently afflicting so many countries might be shed by the development of an 'ecological economics'. □

Band structure of solid inert gases

from B. L. Smith

THE inert gases have long been recognised as useful tools with which to develop the atomic theory of solids, liquids and gases. In the past twenty-five years much progress has been made, but there still remain considerable gaps in our understanding of these 'simple' systems.

In principle, properties of the solidified inert gases, including both thermodynamic and optical behaviour, can be determined by solving the Schrodinger equation, using the total Hamiltonian for all ions and electrons within the solid and their mutual interactions. In practice, the many body problem has to be reduced to manageable proportions by using approximations appropriate to the physical property under consideration. Thus virtually all the different approaches to the problem lead to essentially the one-electron approximation, that is, the band-structure formulation of the electron states, or to the exciton picture of excitations within the solid. A large number of band-structure calculations have now been carried out for the solidified inert gases.

The theoretical predictions can be compared with experimental data by measuring the reflectivity, absorption coefficient and the energy loss function. These, together with more recent determinations of photoemission, allow gap energies and electron affinities to be estimated. Accurate measurements are however extremely difficult because of various technical difficulties encountered in the experiments.

Photoelectron energy distribution curves for the solidified inert gases have recently been determined by N. Schwenter and coworkers at the University of Munich, in collaboration with E. E. Koch of the Deutsches Elektronen Synchrotron (DESY), at Hamburg (*Phys. Rev. Lett.*, **34**, 528; 1975). These measurements represent the first comprehensive set of experimental data for the actual band structure. They are important because they provide direct information concerning the structure of the valence bands, the influence of the conduction bands on the photoelectron energy distribution curves, and some details of electron-electron scattering processes. In particular, the data highlight the discrepancies which exist between the results of various band-structure calculations which have recently been reported.

Measurements were made on films of solid neon, argon, krypton and xenon, 20–100 Å thick, using the DESY synchrotron as a broad-band vacuum

ultraviolet light source. The excitation energy was varied continuously between 8 and 30 eV with a typical resolution of 200 meV. Because the films were very thin, charging of the samples—one of the major problems encountered when investigating photoemission of insulators—was kept to a minimum.

The results provide a wealth of information about the band structure. In the first place, the vacuum level E_V is obtained directly and unambiguously for each solid. These values, combined with data for the gap energies E_G determined from absorption and reflection measurements, allow the electron affinities $E_A = E_V - E_G$ to be estimated and compared with the results of earlier photoelectron yield determinations. For example, E_A for neon is found to be both large and negative (−1.4 eV), which confirms the negative value predicted theoretically, and is consistent with the trend observed experimentally for the other inert gases.

Above threshold, the width of each energy distribution curve increases with increasing photon energy until the photon energy is sufficient to excite electrons from the bottom of the valence band. In this region the width of the curve corresponds to the width of the valence band and is independent of the energy of the exciting photons. Photoemission thus allows the valence band width to be estimated. Schwenter found that the widths of the energy distribution curve increased systematically from neon to xenon as might be expected from the spin-orbit splitting, and were larger than the widths predicted by most band-structure calculations.

There are some interesting anomalies which arise when examining the various band-structure calculations. For example, relativistic calculations for krypton result in a total band width that is 0.5 eV smaller than that observed (2.3 eV), whereas non-

relativistic calculations exceed the experimental width by similar amounts. The best agreement between theory and experiment is obtained by simply adding the spin-orbit energy to the width of non-relativistic bands obtained by Kunz and Mickish (*Phys. Rev.*, **B8**, 779; 1973), but this procedure is somewhat dubious.

Three maxima are noted in typical photoelectron energy distribution curves for xenon but these are not detected for the other solidified inert gases, presumably because of the overlapping caused by the smaller spin-orbit splitting and smaller total band width in the other solids. The shape and relative intensities of the curves are found to depend strongly on the exciting photon energies. Using the experimentally determined parameters for the valence bands, these variations can be used to obtain estimates for the structure of the density of states in the conduction bands. For example, the first maximum of the density of states can be deduced, which corresponds to flat regions of the lowest conduction band. The experimental data thus yield new and important information about the structure of the conduction bands, hitherto unobtainable by other methods.

How do the above calculations and measurements relate to the cohesive energy in the solidified inert gases? It is known that one-electron energies, as obtained for example from Hartree-Fock calculations, over-emphasise Coulomb and exchange terms. More importantly, band energies do not fully include electron-electron interactions. Since electron-electron correlations would be expected to be important in the case of the inert gases, these calculations need some modification to take these effects into account. For example, the basic Hartree-Fock assumption that the ground state wave function can be described as a single Slater determinant of one-particle wave functions can be modified by including excited one-particle wave functions in the combination. But this and other approaches soon run into computational difficulties and still no entirely satisfactory method exists for solving the problem.

Eisenschitz and London carried out the perturbation calculation in 1930 which established the attractive part of the force between two non-polar atoms—the Van der Waals or dispersive term. In the following decade it was confidently predicted that because the atomic polarisability had been linked to interatomic forces, once the structures of the solidified inert gases were established, it would be a relatively simple matter to relate the atomic properties to the bulk thermal and mechanical behaviour.



A hundred years ago

THE preparations for the Geographical Congress in Paris are being actively completed. The large map of France executed by the staff officers will be exhibited, all the sheets having been joined, thus forming one continuous sheet of paper of immense size. The map will be exhibited at the Tuileries in the Salle des États. It will be photographed by the microscopical and panoramic process.

from *Nature*, **12**, 174; July 1, 1875

The past twenty-five years have proved the contrary. Although some very elegant experiments have been performed and a great number of calculations have been carried out, the details, both theoretical and experimental, still prove to be very elusive.

Diamond in a kimberlite xenolith

from G. Malcolm Brown

MOST of the world's diamonds occur as sparsely distributed crystals in the rare, ultramafic potassic rock called kimberlite. The rock is found in pipe-like bodies that reach the present Earth surface, as for example in the major diamond localities of South Africa and Siberia. There is no doubt that the kimberlite material was injected into the crust from deep levels of the Earth, aided in many cases by the explosive forces of a high gas content. The kimberlite matrix has apparently crystallised from a liquid magma, and the fascinating array of rock fragments (xenoliths) embedded in the matrix bear testimony to the source of the magma. These xenoliths are mainly garnet lherzolites, rocks consisting chiefly of garnet, olivine, clinopyroxene and orthopyroxene crystals. Such ultramafic rocks are now widely accepted as being most closely representative of the composition of the Earth's upper mantle. Hence the intrusion of kimberlite has resulted in the transport, to near-surface environments, of parts of the Earth that are otherwise invisible except by indirect geophysical measurements.

Diamonds are not a geologist's best friend. Most minerals occur in a textural association with other minerals such that constraints can be placed on the physico-chemical properties of the whole rock assemblage, and much information can thus be gained about the processes involved in their origin. In contrast, most diamonds occur within the heterogeneous, often fragmentary matrix of the kimberlite pipes and the critical information on temperatures and pressures (and thus depths) of origin can only be inferred from studies on the associated, but generally diamond-free fragments of ultramafic upper mantle (lherzolitic) rock. Another complicating factor is that whereas lherzolite xenoliths occur in many volcanic lavas generated from the upper mantle, they are diamond-free. Hence the genesis of diamond has seemed to be associated specifically with the kimberlite matrix of the diamond pipes, rather than with upper mantle xenoliths as a whole.

Dawson and Smith (*Nature*, **254**, 580–581; 1975) have therefore made an

important recent discovery. They describe the finding of a small diamond crystal (0.8×1.4 mm) in a thin section preparation of a mica-bearing garnet lherzolite xenolith from a kimberlite pipe (diatreme) in Lesotho. C. F. Davidson once wrote that "the richest kimberlites . . . carry one part diamond in 10^7 of rock, and the average is one in 10^{10} or less. So the chances of finding a diamond in a thin section are somewhat around the cube of the probability of finding the needle in the haystack" (in *Ultramafic and Related Rocks*, edit. by Wyllie, 302; Wiley, 1967). Small wonder, therefore, that diamonds are rarely detected other than by the crushing and sorting of huge volumes of kimberlite rocks. Dawson and Smith refer to the few previously recorded occurrences of diamond in eclogite and garnet serpentinite xenoliths from kimberlites, but those xenoliths are of slightly lesser relevance to an upper mantle source material.

The discovery of diamond in the lherzolite permits mineralogists to use the lherzolite minerals in order to estimate the pressure (P) and temperature (T) of formation of the included diamond crystal. In fact, studies of this type have been pursued vigorously since 1973, stemming especially from original work by Boyd and coworkers on the pyroxene mineralogy. Unfortunately, despite a number of sophisticated studies on the phase equilibria and related thermodynamic calculations, the topic is still plagued by doubts as to the dependability of certain data used in the geothermometry and geobarometry estimates. Each new study adds further refinements, though the basic concepts are not questioned, and it is premature for readers to assume that the P - T values given by Dawson and Smith are better than a very broad estimate.

They give valuable mineral analyses and conclude that the assemblage equilibrated at $1,050^\circ\text{C}$, at a pressure of about 4.6 to 5.2 kPa (that is, about 150 km depth). There are, however, several other lines of evidence that need to be considered. Graphite, the other form of carbon in igneous rocks, occurs in some garnet lherzolite xenoliths in kimberlites, as they mention, so the P - T curve for the graphite-diamond transition is relevant in the sense that proximity to this phase boundary is likely. Also, studies of mineral inclusions in diamonds have yielded important data for P - T estimates.

A method of estimating P - T conditions that is more rigorous than analogy with simple synthetic systems is more than overdue. Such an approach, using thermodynamic parameters to formulate a geothermometer based on the iron-magnesium exchange reaction between olivine and calcium-

rich clinopyroxene, has recently been developed by Powell and Powell (*Contr. Min. Petrol.*, **48**, 249–263; 1974). They examine P - T conditions for inclusions in diamond and for ultrabasic xenoliths in kimberlites, and the related studies are cited. Where the P - T lines are drawn for those assemblages, they intersect the graphite-diamond transition line at about $1,250^\circ\text{C}$ and 4.5 kPa. Thus the pressures inferred by Dawson and Smith are corroborated, but the temperature discrepancy is 200°C . In fact, plotting of the Dawson and Smith data (at $1,050^\circ\text{C}$) on the Powell and Powell diagram would suggest equilibration improbably close to the wet pyrolite solidus.

Reference to other methods of estimating P - T conditions, which may or may not be proven better, is made merely to stress the need for caution in accepting specific values at this stage in a rapidly developing science. The major contribution by Dawson and Smith remains an exciting one. They raise other interesting points regarding the stability of phlogopite mica, diamond growth habits, and the possible genesis of graphite by breakdown of diamond. Above all, however, they remind us that the processes of generation of diamond are interwoven with the processes of upper mantle dynamics and magma generation at around 150 km depth in the Earth.

A probe probed

from E. G. Richards

To investigate the structure of DNA you need a probe that will interact with the macromolecule. Electromagnetic radiation has long been a favourite but various chemical reagents are strong contenders. Of these, the simplest is the proton and the next most simple is possibly formaldehyde but in order to interpret the interactions you observe you need to know in detail what is going on. This has hitherto been more than a little obscure in the case of formaldehyde, a matter which has now been put to rights by two elegant and exhaustive papers by McGhee and von Hippel (*Biochemistry*, **14**, 1281 and 1297; 1975).

To begin with, formaldehyde can react with both the exocyclic amino groups of A, C and G residues and the endocyclic imino groups of T and G (and U and I). In each case the result is the substitution of a hydrogen atom by a methylol ($-\text{CH}_2\text{OH}$) group. In the case of the reaction with the amino group, a monoadduct or a diadduct may be formed according to whether just one or both hydrogens are substituted and it would seem that the ghost of the Schiff base has been laid. A further wrinkle in the exocyclic re-

action is provided by the fact that the amino group is coplanar with the heterocyclic ring so that there is the possibility that the monoadduct can exist in two isomeric forms only one of which would interfere with the formation of Watson-Crick base pairs. Experiments with various methylated bases suggested that the more stable isomer precluded base pairs in the case of A and C but not in the case of G residues.

The equilibrium constants for the formation of the various adducts were measured using spectroscopic methods and employing several clever tricks to analyse the data. The formation of the monoadduct in the exocyclic reaction yielded values in the region of 13 M^{-1} while the formation of the diadduct from these gave values some 35 times smaller. The endocyclic reaction gave values of about 2 M^{-1} . Both sorts of reaction were exothermic with enthalpy changes in the range -2 to -6 Kcal mol^{-1} according to the compound studied and the nature of the reaction. Thus at 23°C in 1 M formaldehyde, for example, deoxyadenosine would exist at equilibrium as 25% diadduct, 70% monoadduct and 5% unreacted nucleoside. At higher temperatures one should expect less adduct.

The forward and backward rate constants for adduct formation were also studied. The forward constants for the formation of the monoadduct in the exocyclic reaction turn out to be in the range $0.5\text{--}13 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ which is rather slow. Thus the half life of decomposition of an adenine adduct at 0°C is about 60 h so that dialysis of nucleic acids for a day or two in the cold would be quite inadequate to remove reversibly bound formaldehyde. The rates of these reactions were found to be independent of pH and remarkably insensitive to the addition of compounds such as tetramethyl ammonium chloride, sodium perchlorate or dioxane which perturb the melting of DNA. In another nice point a good linear relation was demonstrated between the logarithm of the rate constant for monoadduct formation of the pK of protonation of the amino group for a long series of substituted anilines and similar compounds. Extrapolation of this relation suggested that the pK of protonation of the amino group in adenine and cytidine was in the range -2 to -3 .

The endocyclic reaction was found to be faster by two or three orders of magnitude and, moreover, pH dependent. At pH 7 there was a similar linear relation between the logarithm of the rate constant and the pK for several nucleic acid base derivatives and also, in the case of 5'TMP, with pH.

All these data are interpreted in

terms of a mechanism wherein a formaldehyde molecule approaches a base in a direction perpendicular to its plane. In the case of the amino group reaction, an intermediate tetrahedral zwitterion is formed which then reverts to the planar adduct. In the case of the imino group reaction the formaldehyde reacts with the negatively charged ionised form and the charged intermediate is then rapidly protonated to form the neutral adduct. In either case the reaction is expected to be inhibited by base stacking such as would be present in a double helix.

Some of the implications of these results to the study of the structure and kinetics of unfolding of DNA are obvious but the further instalments along these lines promised by the authors will be eagerly received.

In a paper containing less physical chemistry but more immediate relevance to nucleic acid structure, Rhodes (*J. molec. Biol.*, **94**, 449; 1975) describes the reaction of methoxyamine and carbodiimide with yeast tRNA^{phe}. The first of these reagents is specific to C and the other to U and G; contrary to some earlier reports it was also found to react with dihydrouridine. Using fingerprinting techniques she was able to determine which bases in the sequence were attacked and it is gratifying that the results are wholly consistent with the three dimensional structure of this tRNA species as recently revealed by X-ray diffraction. This was so even in the presence of spermine as used in the crystallisation brew.

It has always been a worry that the packing of the tRNA molecules into the crystal lattice could result in their distortion into biologically irrelevant shapes. Rhodes's work goes some way to allaying such anxieties.

Xeroderma pigmentosum and light-induced cancer

from F. Giannelli

THREE recent papers in the *Proceedings of the National Academy of Science* have set the spotlight on Xeroderma pigmentosum (XP), a rare recessive disease which seems to hold the answer to many questions on the genetics and physiology of DNA repair in man and on its role in cancer and development.

Patients with this disease show, soon after birth, hypersensitivity to sunlight gradually followed by regressive changes and multiple recurring neoplasms in the exposed parts of the skin. They may also present neurological abnormalities which in approximately 14% of the cases are grossly incapacit-

ating and progressive.

In 1968 Cleaver discovered that XP cells are deficient in excision repair, a DNA repair system which, by a series of enzymatic steps, excises DNA lesions caused by ultraviolet light or similar mutagens, and replaces them with patches of DNA newly synthesised using the undamaged strand as a template. It seemed reasonable at that time to suggest that unrepaired DNA damage was a sufficient and necessary cause of light-induced cancer. Since 1971, however, patients with typical features of XP but normal excision repair (XP-variants) have been observed and, it seemed, therefore, appropriate to retreat to a less dogmatic view and postulate simply that unrepaired damage was probably a sufficient though possibly not a necessary cause of actinic cancers. For four years the XP-variants have been an uncomfortable puzzle, but now Lehmann *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 219; 1975) have succeeded in showing that cells from such patients are defective in a process (post-replication repair) which enables normal cells to avoid reproductive death by overcoming the obstacles to DNA replication caused by uncorrected DNA damage. Lehmann *et al.*'s (1975) findings are in keeping with the observation that XP-variants are less efficient than normal cells in the reactivation of ultraviolet-irradiated infecting adenovirus 2 (Day, *Nature*, **253**, 748; 1975) and clearly indicate that the XP phenotype is the direct consequence of a DNA repair defect.

As so often happens in science, when one problem is solved, another appears.

For many years it has been thought that placental mammals, and man in particular, are deficient in enzymatic photoreactivation—a repair process catalysed by an enzyme which, if activated by light of the appropriate wavelength, splits the covalent bonds whose formation between adjacent pyrimidines of the same DNA strand is induced by ultraviolet irradiation (Cook, J. S., in *Photophysiology*, edit. by Giese, A. C., **5**, 191; 1970; Trosko, J. E., and Isoun, M., *Int. J. Radiat. Biol.*, **18**, 271; 1970). Recently, however, Sutherland and collaborators in a series of papers have presented evidence to show that photoreactivating enzyme is present in human leukocytes and fibroblasts, plays a part in the reactivation of infecting ultraviolet damaged Herpes Simplex virus, is active also on the DNA of living cells and is absent or markedly reduced in excision proficient and defective XP cells strains (*Nature*, **248**, 109; 1974; *Biochemistry*, **13**, 4710; 1974; *Nature*, **254**, 627; 1975; *Proc. natn. Acad. Sci. U.S.A.*, **72**, 103; 1975).

The findings of Sutherland *et al.* in XP are unexpected and pose many

intriguing questions. For example, is the defect in photoreactivation secondary and incidental to defects in dark repair or does it play an important part in the development of the XP phenotype, a view favoured by Sutherland *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 103; 1975). If the latter were true an inverse relationship could be expected between the severity of the photoreactivation and the dark repair defects observed in different patients but this is not yet borne out by the available data. The constant association observed between photoreactivation and dark repair defects in XP is difficult to explain in simple genetic terms. For example, the hypothesis that the two defects are inherited as independent mutations could make sense only if defects in one repair system alone were insufficient to produce the XP phenotype and if the mutations affecting photoreactivation and both dark repair systems (excision and post-replication) were very closely linked. This seems unlikely especially if one considers the genetic heterogeneity of excision-defective XP. So far five complementation groups and two types of XP showing distinct complementation kinetics have been recognised and it seems possible that as many as five loci are involved in this disease in spite of the apparent similarity of the biochemical defect in the different complementation groups (Kraemer *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 59; 1975; Bootsma *et al.* in *Molecular Mechanisms for the repair of DNA*, edit. by Hanawalt and Setlow; Plenum Press, New York, in the press; Giannelli and Pawsey, *J. Cell. Sci.*, **15**, 163; 1974 and unpublished).

As an alternative explanation for their findings Sutherland *et al.* (1975) suggest that the mutations in XP affect a regulatory gene common to photoreactivation and dark repair, but this explanation too is difficult to reconcile with the complementation data. May one wonder, then, whether the proteins defective in XP act as or, perhaps more likely, favour the function of the photoreactivating enzyme? Might many of the enzymes of the DNA repair systems be cointegrated to form a complex whose diverse functions could be differentially upset by mutations in different component proteins?

In spite of present uncertainties, XP clearly demonstrates the importance of DNA repair and its relevance to oncology. It offers an opportunity to study the genetics, physiology and biochemistry of DNA repair in man and, in addition, by drawing our attention to photoreactivation and to different dark repair pathways, it sets us the task of determining the relative con-

tribution of these processes to cellular genetic homeostasis.

Much still needs to be done to clarify the genotype-phenotype relationships in XP. Of particular interest are the neurological abnormalities, which seem to result from diffuse and premature neurone loss. This process may provide clues to understand the ageing of non-regenerating tissues and organs such as the brain. Does, for example, the absence of neurological abnormalities observed so far in XP-variants, suggest that excision repair is of primary importance for non-regenerating tissues and consequently that DNA damage has a key role in ageing (see Burnet, *Intrinsic Mutagenesis: A Genetic Approach to Ageing*; Medical and Technical, Lancaster, 1974)?

The lithosphere as prime mover

from Peter J. Smith

ALTHOUGH the broad tenets of plate tectonics are now widely understood and accepted, there is still ample scope for the investigation of, and disagreement over, the all-important details. One of the most crucial of these 'details' is the precise mechanism which allows continents to drift, sea-floors to spread and hence plates to interact. When the geophysicists of the 1940s and 1950s finally broke the chain of disbelief in Wegenerian drift by proposing the existence of convection currents in the Earth's mantle, they had in mind a system in which the relevant parts of the overlying crust or lithosphere were carried along as the passive partners of a self-contained driving force below. This idea was then later modified to enable the lithosphere to become an integral, albeit still passive, component of the convection pattern. More recently, however, some workers have suggested that the lithosphere is not only a part of the convective cycle but the active part. According to this view, either the cold lithosphere descending at subduction zones pulls the plate or the hot lithosphere created at ridges pushes it—and the asthenosphere merely acts as a counterbalancing channel for the flow of material in the opposite direction.

Which, if any, of these mechanisms actually operate (they are not mutually exclusive) is anyone's guess at present; but in an attempt to reduce the options Harper (*Geophys. J.*, **40**, 465; 1975) has constructed a model which enables certain plate parameters to be predicted and hence compared with observed values. Harper's basic premise is that, irrespective of what the principal driving mechanism is, subduction zone pull and oceanic ridge push must

play some part. His model then assumes that these are the only driving forces and that lithospheric motion is resisted only by Newtonian viscous drag in an asthenosphere in which the return flow is everywhere antiparallel to the plate velocity.

The first prediction of the model is that, if the commonly accepted ('reasonable') values of density, viscosity and plate thickness are correct, a typical subduction zone pulls about seven times as hard as a typical oceanic ridge pushes. This is not a conclusion which can be tested directly, of course; but it is an interesting one nonetheless, especially as it is likely to confirm many an intuitive belief based simply on the relative scales of subduction zones and oceanic ridges.

More importantly perhaps, Harper's model permits estimation of the angular velocities of plates, from which relative angular velocities between plate pairs may be calculated. As far as pairs among the Antarctic, Pacific, Indian, African, American, Eurasian, Nazca and Cocos plates are concerned the agreement between the relative angular velocities predicted from the model and those observed—as quoted by Chase (*Geophys. J.*, **29**, 117; 1972)—is regarded by Harper as no more than 'tolerable' in most cases, and for the Cocos plate in particular rather bad. The Cocos example is instructive, however, in that it leads to a refinement of the original model which not only brings the Cocos plate into line with the others but improves the agreement between prediction and observation in all cases. There are several possible problems with the Cocos plate, one of the most obvious being that it is small. But it is also relatively thin. When the relative thicknesses and the thickening with age of all plates are taken into account, predicted relative angular velocities for the various plate pairs range from 0.81 to 1.38 of the observed velocities (compared with 0.80 to 8.8 for the unmodified model and 1.0 for perfect agreement).

Considering the simplicity of the model (even the modified model), the approximations incorporated into it and the uncertainties in the numerical values adopted, the agreement between prediction and observation is really quite remarkable. Indeed, Harper himself seems somewhat surprised, arguing that coincidence cannot be entirely excluded. But taken at face value, the results suggest that the motions of the eight principal plates can be largely accounted for without considering any driving forces but the downward pull at subduction zones (especially) and the outward push from oceanic ridges.

In addition, as a result of considering the stresses acting within tectonic plates, Harper is able to throw some

possible light on to the vexed question of why and how the ancient supercontinents split apart in the first place. Prior to the opening of the Red Sea, the eastern boundary of the then African-Arabian plate would have had a re-entrant corner to the east of the tip of what is now the Somali Republic. According to Harper such a corner would magnify the (tensile) stresses locally, facilitating the formation of a crack. Certainly a crack developed in this case, propagating towards the southern tip of Arabia and then, with a change of direction, up what was to become the Red Sea (a course presumably dictated partly by local geology and partly by the forces pulling towards the subduction zone beneath the Zagros mountains in the north-east). But this change of direction would have produced a new re-entrant corner at the southern tip of Arabia, a new local concentration of stress and (as evidently occurred) a new split forming the east African rift.

Would it be possible to explain the gradual break up of Gondwanaland (or even Pangaea) entirely in terms of a series of cracks propagating from re-entrant boundary corners, perhaps aided in many cases (once the process got going) by pulling at appropriately situated subduction zones?

Faithful transcription *in vitro*

from T. Barrett

RECENTLY much interest has focused on reproducing *in vitro* the transcriptional ability of native chromatin. The manipulation of chromatin during its isolation, most notably shearing to render it soluble, is liable to alter its template activity, and it is reasonable to be sceptical about the relationship that *in vitro* transcription products bear to RNA synthesised *in vivo*. De Pomerai *et al.* (*Eur. J. Biochem.*, **46**, 461; 1974) demonstrated that chromatin prepared by various published methods differed both in structure, as defined by the DNA:RNA:protein ratio, in template activity using both endogenous and added homologous RNA polymerase, and in the size of transcript produced. Shearing of chromatin resulted in a decrease in transcript size and an altered size distribution on formamide sucrose gradients. These authors suggested that extraction of chromatin and RNA polymerase from eukaryotic cells may remove factors necessary for *in vivo* transcription and subsequent processing of RNA.

More recently Noll *et al.* (*Science*, **187**, 1203; 1975) demonstrated that the structure of native chromatin is easily destroyed when chromatin is prepared by conventional methods involving

shear. Such chromatin does not have the repeating structure shown by native chromatin when digested by micrococcal nuclease. They have developed a technique whereby the native structure is retained, which involves slight nuclease digestion of isolated nuclei followed by lysis in hypotonic buffer. The resulting chromatin shows the same 200 base pair repeating unit found in nuclear chromatin. But they point out that chromatin, even when prepared by this comparatively gentle method, is not necessarily identical with chromatin in the nucleus.

The most direct approach to the study of fidelity of transcription *in vitro* has been to search for specific RNA sequences in the transcription product. Paul and Gilmour (*J. molec. Biol.*, **34**, 305; 1968) first indicated that organ-specificity was retained in isolated chromatin, but their hybridisation techniques only provided information about RNA transcribed from reiterated DNA sequences. The use of reverse transcriptase to copy specific messenger RNAs into highly radioactive complementary DNAs has, however, made it possible for Axel *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 2029, 1973) and Gilmour and Paul (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 3440; 1973) to demonstrate that RNA transcripts of chromatin isolated from erythropoietic tissues contain globin-specific RNA sequences.

These studies were performed using bacterial RNA polymerase and indicated nothing about the accuracy of transcription of the globin genes on isolated chromatin. Steggle *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 1219; 1974) compared the transcription of globin-specific RNA sequences on rabbit bone marrow chromatin using both bacterial and mammalian form B RNA polymerase. Both enzymes transcribed globin-specific RNA sequences, but with mammalian enzyme globin sequences represented a higher proportion of the RNA synthesised (0.05% for mammalian as opposed to 0.016% for the bacterial enzyme). The use of homologous rather than bacterial polymerase, however, does not provide the complete answer to the problem of accurate *in vitro* transcription. Honjo and Reeder (*Biochemistry*, **13**, 1896; 1974) found that added homologous RNA polymerase transcribed the rRNA genes of *Xenopus laevis* chromatin in an aberrant manner. The polymerase transcribed both strands and the spacer regions and in fact was no more accurate than bacterial polymerase in this respect.

In a recent paper Marzluff and Huang (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 1082; 1975) claim to have achieved faithful transcription of low molecular weight RNA species using the endogenous RNA polymerase of mouse

myeloma chromatin. Chromatin was isolated from nuclei by lysis in hypotonic buffer and then sheared by passing it through a narrow gauge syringe needle. The accuracy of transcription of the 5S RNA and tRNA genes *in vitro* was confirmed by the correct size of the transcripts, 5S for the 5S gene and 4.5S for the precursor of 4S tRNA. In addition correct strand transcription was indicated for the 5S RNA gene since the transcript hybridised only to the + strand of *Xenopus laevis* 5S DNA. Although Zylber and Penman (*Proc. natn. Acad. Sci. U.S.A.*, **68**, 2861; 1972) and Reeder and Roeder (*J. molec. Biol.*, **67**, 433; 1972) had previously shown that rRNA is faithfully transcribed in isolated nuclei, it is of interest that in isolated chromatin where half the nuclear protein and 90% of the nuclear RNA is removed, an accurate transcript of the 5S and 4S genes can still be obtained. There are differences, however, between isolated nuclei and isolated chromatin. Marzluff and Huang found that the chromatin system was much less stable as regards RNA synthetic activity. Activity is rapidly lost at 4 °C and is completely destroyed by freezing. The average molecular weight of the total RNA transcript from chromatin is lower, probably because of the shearing process employed during chromatin preparation, although this does not appear to affect the low molecular weight RNAs which are still synthesised accurately. In chromatin no 4S RNA was identified, although a 4.5S precursor species was synthesised. This contrasts with the nuclei where some 4S RNA is produced and suggests that the enzyme responsible for cleaving the 4.5S precursor is missing or inactive in the chromatin preparation.

In spite of the differences, the preferential incorporation of γ -³²P-GTP into 5S and 4.5S RNA suggests that these species are being reinitiated and transcribed continually *in vitro*. The genes for 5S and 4S RNA represent only a small portion of the nuclear DNA (0.001% and 0.005% respectively) but they account for nearly 1,000 times that amount of RNA, indicating that strict template restriction is maintained *in vitro*. Marzluff and Huang suggest that the structure of these genes in chromatin and their interaction with factors other than polymerases may result in their great activity *in vitro*. This may explain why isolated polymerases, whether homologous or bacterial, do not show the same extensive restriction when used to transcribe chromatin. Such studies emphasise that the native structure of chromatin and its intimate association with some, as yet unknown, labile factors may be absolutely essential for the accurate transcription of genes *in vitro*.

review article

Non-Darwinian "evolution" and biological progress

J. M. Thoday*

The use of terms such as "higher" and "lower" to describe different forms of life shows that we tend to think of evolution not only as adaptive, but also in some sense as progressive. That sense can be defined in a way that makes it possible to show how some "neutral" polymorphisms could contribute to progressive evolution.

PROGRESS and its adjective progressive are ambiguous words. They may imply merely unidirectional change, or they may imply change in a direction that is regarded as improvement. They have been used in both senses (sometimes both senses are confounded) in writings about evolution, but though there have been difficulties in defining progressive evolutionary change in the sense of improvement, many authors have clearly felt that evolutionary changes have given rise to improvement. Indeed most feel so: otherwise the use of higher and lower as applied to various animal and plant groups would be seen to be empty of meaning.

Some time ago^{1,2}, I made an attempt to define "biological progress" in a way that I thought avoided the circularity of the arguments that had been used by Julian Huxley^{3,4} whose definition of progress was based on the implicit assumption that progress has in fact occurred. Stebbins' recent book⁵ also makes this implicit, and it seems to me, unwarrantable assumption.

My attempt avoided this assumption by defining progress solely in terms of the general conditions of survival. Most of the trends Huxley and Stebbins identified as progressive fall into progress as I defined it either under the headings of phenotypic flexibility (see below) or environmental control.

Current controversy about the role of neutral alleles in evolution prompts me to consider to what extent such neutral alleles may contribute to progressive evolution as so defined.

Neutral alleles and adaptive evolution

The controversy concerning neutral alleles centres on the question of the proportions of genetic differences produced by mutation that can be regarded as selectively neutral, and on the extent to which such spread of neutral alleles may explain differences between populations and species and also explain the number of alleles at a locus to be found in a population. Since the spread of neutral alleles is a purely chance process, such evolution has been called non-Darwinian⁶.

The theory of evolution has two major components, the concept of evolution itself and the mechanism of change that has brought evolution about.

The concept of evolution explains the classifiability of

organisms, the facts of plant and animal geography, the common behavioural, morphological, embryological, anatomical, physiological, biochemical, cytological and genetical properties of diverse organisms, the facts of palaeontology and palaeobotany, results of the study of microevolution in "nature" and in the laboratory, and the results of plant and animal breeders. It does so by postulating that diversity is the consequence of modification over the generations of differing lines descended from common ancestors. It does not explain adaptations as such but explains the diversity of adaptations.

Our concepts of the mechanism of evolution seek to explain the change that gave rise to this diversity of adapted organisms, and here it is essential that an evolutionary theory must explain adaptedness and changes of adaptive mode. Natural selection is the agent central to Darwinian evolution because it explains adaptedness. Modern Darwinian theory has the following components: mutation, random with respect to place, time and need, which provides the source of allelic heterogeneity that is the basis of change; segregation and recombination that provide varying combinations of alleles and diversity of genotypes; selection which increases the relative frequency of the genotypes that on the average lead to the fitter (relatively better adapted) phenotypes; and genetic drift which leads to random changes in allele frequency and in small populations may establish an allele in a population sometimes even if that allele leads to phenotypes less fit than some alternative allele and which will certainly establish neutral alleles. Those of strong selectionist views would allow only a very minor role for drift, except in its special forms of "founder principle" and when there are very restricted bottlenecks in population size.

Non-Darwinian evolution, on the other hand, would give drift a major role in molecular evolution by postulating that a large proportion mutations leads to neutral alleles, that is to say to alleles that do not differ in their effect on fitness from the alleles that mutated.

Two interesting points emerge. One is that these new developments would greatly increase the role of chance in the determination of the direction of evolution. Darwinian evolution involves considerable elements of chance, not only with respect to drift, but also to the timing and direction of mutation and the chance involved in the origin of particular combinations of genes. It is of interest here to draw attention to the fact that it is this chance component of evolutionary theory to which many (see for example ref. 7) object, with its consequences that the precise course of evolution is unpredictable, that evolution could have

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taken many other paths, that any particular species or individual must be regarded as an accident, and that evolutionary changes are not necessarily either adaptive or progressive; these are aspects of the theory that do not conform with any of our fundamental philosophies or with our egocentric outlook. Non-Darwinian evolution would simply add a greater component of chance.

The second point is that in so far as substitution of neutral genes by chance processes is regarded as "evolution" it must be questioned whether the "evolution" it considers is the same as "evolution" considered in the Darwinian sense.

Population geneticists have come to talk of changes of allele frequencies as evolution. But evolution, in the classic sense, involves adaptive changes of phenotype. Neutral allele substitutions cannot be adaptive, and, from the point of view of adaptive evolution theory, it seems doubtful whether a genotypic difference which can never make any difference to fitness is to be regarded as relevant. The relevance to adaptive evolution of a genotypic difference which makes a difference that we can detect at some level we call phenotype, but which makes no difference to the economy of the organisms, is questionable.

In discussing whether "neutral" alleles might in any sense be relevant to adaptive evolution we must therefore consider the kinds of neutral allele pairs we may envisage. There are four, of which we shall see that only two, conditionally neutral and pseudo-neutral allele pairs, can be envisaged as playing any role in adaptive evolution.

Strictly neutral allele pairs

These are pairs of alleles that can in no circumstances make any relative difference to fitness and therefore can in no circumstances contribute to adaptive or progressive evolution. They may include redundant codon differences, though it should not be forgotten that these might differ in efficiency of transcription or replication, and that some redundant codon pairs may involve some recombinational load, for example UUA and CUU both code for leucine but can recombine to give UUU phenylalanine.

They may also include allele pairs that are meaningful codon differences but lead to "redundant" amino acid substitutions in parts of a protein that do not affect the protein's function. Some of these differences may be detectable by techniques other than sequencing, for example tests of electrophoretic mobility, heat stability, enzyme activity, and thus have some detectable effect on some aspects of phenotype, but might nevertheless have no effect on fitness. It seems fair to suggest that the more readily detectable at such levels the differences are, the more likely they are to affect fitness.

Among these differences there may be some that have an overt effect on gross phenotype. The history of population genetics, in which one after the other overt polymorphism has been claimed to be neutral but has subsequently been proved to be maintained by selection, should lead to some scepticism concerning this possibility.

Quasi-neutral allele pairs

These have been proposed by Kimura, and the proposition might be regarded as a retreat from the more strictly neutralist position though he originally⁸ did refer to "neutral or nearly neutral" alleles. They are alleles that affect fitness, but do so to such a small extent that their fate can be accurately predicted by neutral theory. Unless, however, their negligible effect on fitness can change (in which case they are conditionally neutral, see below) they are as unlikely to be relevant as strictly neutral alleles.

Conditionally neutral allele pairs

These are allele pairs that were neutral or quasi-neutral in past circumstances but may be non-neutral in some future genetic or external environmental circumstance. To separate these as a category is important for they may be envisaged as a class of neutral alleles that may be established and maintained in a population without affecting fitness, but in some other circumstances might form a source of genetic variance that through selection could improve adaptedness.

As an illustrative model example we might envisage a pair of alleles differing in the heat stability of the enzyme they code for: say that one enzyme is inactivated at 33 °C, the other at 40 °C. Assuming that, below the critical temperature 33 °C, the isoenzymes have the same biochemical properties, and also assuming that the environment of the population never involves temperatures higher than this, the alleles will be neutral. But if the environment changes, or if some part of the population migrates to some new area, by accident or as a result of change elsewhere in the genome that enables the population to spread out of its hitherto restricted range, so that temperatures above 33 °C are now met, then the alleles cease to be neutral and natural selection will begin to affect their frequencies. The conditionally neutral polymorphism might then be said to be preadaptive.

Pseudo-neutral allele pairs

These are of two kinds. The first are similar to conditionally neutral alleles. They are those which were in the past non-neutral but, because conditions have changed, are neutral now. They must of course contribute to the pool of conditionally neutral alleles now since the change of conditions might be reversed, but they must be distinguished because their presence in the population is a result of selection so that they, being neutral now, will contribute to the difficulty of determining what proportion of molecular polymorphism results from non-Darwinian evolution.

The second are alleles which are neutral or quasi-neutral in present circumstances because selection has made them so. Among these we include those showing frequency dependence, for by definition these at equilibrium are neutral, but their neutrality is itself a consequence of selection. Cyclic selection, niche-dependent disruptive selection, especially with habitat choice⁹, and density dependent selection may all contribute to such pseudo-neutrality. Balanced polygenic systems as envisaged by Mather would also involve pseudo-neutrality, the relative fitness effects of alleles at the separate loci being neutralised by their balancing with alleles of opposite effects in linked loci. Modifiers may fall into this category also.

There is good evidence that some molecular differences are subject to such frequency dependent selection. (See Kojima and Yarbrough¹⁰ for direct frequency dependence, Powell¹¹ or Ayala¹² for niche-dependent disruptive selection.) Furthermore we have no reason to suppose that many of the molecular differences we can detect by such techniques as electrophoresis are not part of polygenic systems, just as may be differences in reiterated DNA.

Pseudo-neutral genes are clearly subject to Darwinian evolution.

Neutral alleles and progressive evolution

In considering the possible contribution of neutral alleles to progressive evolution we must have in mind the changes that fall under the definition of progressive changes.

To survive, in anything but the short term, a population or species must have in sufficient measure each of

three properties, adaptedness, stability and variability.

These three concepts express the three basic conditions of continued life, and two of them, genetic stability and variability, are in part antithetic, for they express the need to maintain present adaptedness through stable heredity, but at the same time to preserve adaptability to future different environments.

It needs to be stressed first that we are by no means solely concerned with inorganic sources of environmental change, for evolutionary change of one species is an important environmental change for others that interact with it. Second that, at least from the point of view of the organisms, future environmental change, and hence the adaptation that will be required, is unpredictable. Adaptation to the unpredictable requires the generation of variance at random¹³, but to generate variance at random necessarily involves a reduction of genetic stability. We can therefore see that progressive evolution must be evolution that leads either to a reduction in the need to generate random variance, or to the storage of such variance in temporarily ineffective forms.

In my original discussions of this concept of progress^{1,2}, I pointed to various ways in which these ends have been achieved. These can be summarised under the headings of the various components of long term fitness as follows.

(1) Adaptedness: this expresses the degree to which individuals fit contemporary environments, and within populations relative adaptedness is equivalent to Darwinian fitness. When we are comparing species or major groups, however, since most of them survive well in their contemporary environments, we cannot look to adaptedness in this sense when looking for evidence of progressive evolution.

(2) Genetic stability: this expresses the capacity of individuals to produce adapted offspring, and is promoted by asexual reproduction, inbreeding, homozygosity, and low mutation rates.

(3) Variability: this expresses the capacity of a population to change and depends on (a) genetic flexibility, which is the capacity of the population to produce offspring different from their parents, and is promoted by high mutation rates, heterozygosity and outbreeding; and (b) phenotypic flexibility. Phenotypic flexibility expresses the range of environments in which the individual can function, or the ability of the individual to "recognise" and move from unfavourable to favourable environments. It is this component of fitness that covers most of the progressive trends recognised by Huxley³ as making organisms relatively independent of the environment, or rather of certain aspects of environmental variation. Some would include phenotypic flexibility as part of adaptedness, but I separate them because such flexibility is part of adaptability. Recent developments show that relatively "simple" organisms have more phenotypic flexibility than might hitherto have been thought for the gene control systems of, for example, *E. coli*¹⁴ or *Aspergillus nidulans*¹⁵ provide efficient biochemical flexibility allowing the individual to adapt to variation in the external environment.

(4) Stability of environment: this determines the need for change which can be reduced by (a) specialisation for stable components of environment, a risky process because the continued stability of the habitat is unpredictable, and (b) control of environment directed towards maintaining its suitability, or making it more suitable and keeping it so.

It is the changes that may lead to partial resolution of this antithesis between the requirements of stability and variability that we must define as progressive changes. The antithesis lies between genetic stability and genetic flexibility and is resolved by any increase of phenotypic flexibility, by the adoption of a stable habitat, or of environmental control (which requires the development of communities and, in the ultimate, ability to predict in order to correct environmental trends).

These are ways that reduce the need for genetic flexibility. But the direct antithesis between genetic stability and genetic flexibility has also been reduced by two developments, and may possibly have been reduced by a third.

The first is through the adoption of dual breeding systems, asexual reproduction providing stability and sexual reproduction providing flexibility.

The second is through the development of genetic systems that permit the storage of potential variation^{16,17} without consequent variation of fitness. Such storage is achieved through dominance in diploids, the heterozygous recessives being a source of variance that is "hidden". In diploids and haploids, balanced polygenic systems provide a source of stored potential variation. In outbreeding populations the fixed variation is freed at a rate determined largely by recombination. In inbreeders, complementary alleles may be fixed in different more or less homozygous lines, and the freeing of this variation depends on the frequency of hybridisation (outbreeding) between such lines and on the subsequent recombination in the hybrids. Recombination frequencies are of course also under genetic control, which may perhaps be relaxed in unfavourable circumstances, after wide outcrossing, or as a result of intensive selection, so that the rate of release of potential variation may itself be increased when genetic flexibility is most needed.

The third possibility is that in unfavourable circumstances the control of replication, including DNA repair systems, may be impaired, thus leading to increase in mutation rate when genetic flexibility is most needed¹⁸.

It is clearly in respect to the resolution of this antithesis between genetic stability and genetic flexibility, that neutral allele polymorphisms might be important.

Strictly neutral allele pairs clearly make no contribution to short term or long term fitness, and if they exist they are mere evolutionary noise.

On the other hand those conditionally neutral allele pairs that have been established in populations by non-Darwinian evolution, but may in some future conditions cease to be neutral, provide in principle a potential source of genetic flexibility that does not involve loss of genetic stability. They thus provide an additional source of stored potential variation. Polymorphisms for such conditionally neutral alleles may therefore provide a source of adaptation to the unpredictability of the environment. If so, non-Darwinian evolution is an additional source of evolutionary progress.

I conclude that neutral allele pairs can only contribute to evolution in the classic sense, as distinct from evolution defined as mere gene frequency change, if the neutrality is "conditional". The establishment of unconditionally neutral alleles is mere evolutionary noise. The establishment by "non-Darwinian evolution" of polymorphisms for neutral alleles which may in future combinations or conditions cease to be neutral may provide an important source of genetic flexibility that does not impair genetic stability. The establishment of such polymorphisms would be an additional form of progressive evolution.

- ¹ Thoday, J. M., *Symp. Soc. exp. Biol.*, 7, 96-113 (1953).
- ² Thoday, J. M., in *A Century of Darwin* (edit. by Barnett, S. A.), 313-333 (Heinemann, London, 1958).
- ³ Huxley, J., *Evolution* (Allen and Unwin, London, 1942).
- ⁴ Huxley, J., *Evolution and Ethics* (Pilot Books, New York, 1947).
- ⁵ Stebbins, G. L., *The Basis of Progressive Evolution* (University of North Carolina Press, Chapel Hill, 1969).
- ⁶ King, J. L., and Jukes, T. N., *Science*, 164, 788-798 (1969).
- ⁷ Koestler, A., *The Case of the Midwife Toad* (Hutchinson, London, 1971).
- ⁸ Kimura, M., *Nature*, 217, 624-626 (1968).
- ⁹ Thoday, J. M., *Proc. R. Soc.*, B182, 109-143 (1972).
- ¹⁰ Kojima, K. I., and Yarbrough, K. M., *Proc. natn. Acad. Sci. U.S.A.*, 57, 645-649 (1967).
- ¹¹ Powell, J. R., *Science*, 174, 1035-1036 (1971).
- ¹² McDonald, J. F., and Ayala, F. J., *Nature*, 250, 572-574 (1974).
- ¹³ Thoday, J. M., *Theoria to Theory*, 2, 29-38 (1967).
- ¹⁴ Jacob, F., and Monod, J., *J. molec. Biol.*, 3, 318-356 (1961).
- ¹⁵ Cove, D. J., and Pateman, J. A., *Nature*, 215, 1234-1237 (1967).
- ¹⁶ Mather, K., *Biol. Rev.*, 18, 32-64 (1943).
- ¹⁷ Mather, K., *Genetical Structure of Populations* (Chapman and Hall, London, 1973).
- ¹⁸ Sturevant, A. H., *Proc. natn. Acad. Sci. U.S.A.*, 25, 308-310 (1939).

articles

An electrical conductivity anomaly and rifting in southern Africa

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Measurements of electrical conductivity provide information about rifting in eastern and southern Africa. Overall geophysical and geological evidence supports the view that the conductive zone marks an extension of the African Rift system along old weak zones in the lithosphere.

A MAGNETOMETER array study in 1972 (J. H. de B. *et al.*, unpublished) discovered a zone of concentrated induced electric currents at lithospheric depths crossing South-west Africa, Botswana and western Rhodesia. The array of 25 three-component magnetometers gave results from which three periods of magnetic disturbance were analysed. Magnetograms and maps of Fourier transform amplitudes and phases show a large anomaly in the vertical and northward horizontal variation fields over the period range 21–171 min. A well defined reversal in the vertical field is observed in South-west Africa between 20°S and 21°S. Transfer functions between mean horizontal field components and the vertical component show a conductor that trends east–west in the western part of the array and curves north-eastward in the eastern part. The half-widths of normalised anomalous fields and the induction vectors indicate that the conductor is at crustal depth. In South-west Africa the conductor is well covered by the array and is no more than 100 km wide (Fig. 1). In Botswana the conductive zone is wider and its limits are less adequately defined by the array. A south-westward continuation is possible. At the north-east limit of the array its maximum width is about 250 km (Fig. 1). From east to west the conductive zone continues the south-westward line of the Luangwa–middle Zambezi Rift (Fig. 2) and bends south of the Okavango Delta to run nearly westward across South-west Africa (Fig. 1).

To test the hypothesis that the conductive zone indicates a continuation of the known rift system of Africa, we note other relevant evidences of such a continuation. In north-eastern South-west Africa and all of Botswana the Kalahari sands of Tertiary or younger age cover the hard rocks and hide almost all tectonic features¹. Boreholes reveal Mesozoic Karroo sediments or volcanics under much of the Kalahari basin² (Fig. 1) but have not so far penetrated the recent sediments in the Okavango Swamps (D. Hutchins, personal communication). Major linear features with a north-east trend are found near the Okavango and have been interpreted as faults^{2,3}; they continue the line of the middle

Zambezi–Luangwa Rift. The middle Zambezi valley, containing Lake Kariba, is an asymmetric rift preserving Karroo rocks downfaulted between Precambrian shield rocks; its fault pattern is reasonably well known^{4,5}. Details of the Luangwa Rift faults are lacking⁶ but the valley floor is largely covered by Karroo sediments and several igneous centres are known⁴, including Cretaceous carbonatite volcanoes^{6,7}. Carbonatites and kimberlites occur also in the middle Zambezi⁸ and kimberlites near Orapa in Botswana (Fig. 1). As early as 1921 Gregory⁹ considered the Luangwa–middle Zambezi Rift as an extension of the East African rift systems better developed and known in Kenya and Ethiopia. Others^{7,10} have already used geological arguments to propose south-westward continuation of the Luangwa–middle Zambezi Rift into Botswana.

Seismological evidence

Considerable seismic activity with magnitudes up to 6.7 occurred in the Okavango Delta region in the period 1949–55 with 90% of events in late 1952–early 1953 (refs 11 and 12). Reeves² mapped 50 earthquakes in Botswana over the period 1965–71, and found a concentration of epicentres in the Okavango Delta and scattered events in the central Kalahari, with the broad Ghanzi Ridge of post-Damara sediments between the two seismic regions (Fig. 1). In the Lake Kariba region a low initial level of seismicity rose several orders of magnitude as the lake filled, culminating in three events of magnitudes near 6 in 1963 just after the lake level peaked. Triggering of renewed activity on the existing faults, by incremental solid stress rather than water pressure, was suggested by Gough and Gough⁵. Since 1963 activity has continued with very slow if any decline, in a manner consistent with the hypothesis that the region is now seismic in a steady state. Mechanisms for two of the large events show normal faulting with one nodal plane striking N25°E parallel to regional faults^{13,14}. These and other fault plane solutions for earthquakes in eastern and southern Africa^{13,15} suggest tensile deviatoric stress in a WNW–ESE direction. Fairhead and Girdler¹³ showed that in the period 1963–70 earthquakes in southern central Africa were located in a broad seismic zone running southward from Lake Tanganyika to a point just west of Lake Kariba. Except in the Lake Kariba area the middle Zambezi–Luangwa Rift was virtually aseismic through 1963–70.

Epicentres from several sources (refs 16–18 and L. M. Fernandez and J. A. Guzman, unpublished) for the period

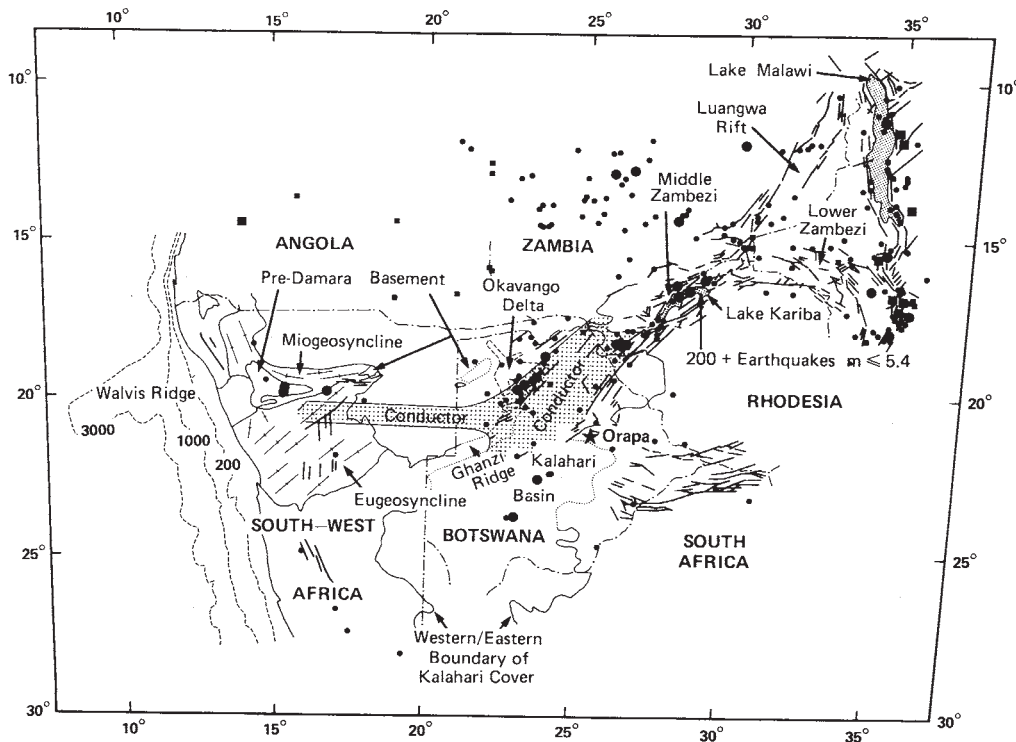


Fig. 1 The conductor in relation to seismicity and tectonics of southern central Africa. Small dots, $2 \leq m \leq 4$; large dots, $m > 4$. Small squares, intensities < 3 ; large squares > 3 . Faults (bold lines) are from refs 3, 6, 40-42. Fold axes (with crossbars) are from ref. 1. Geological boundaries are from refs 1, 3 and 40. Offshore depths are in metres.

1965-73 have been plotted in Fig. 1. Their distribution supports the hypothesis that the Luangwa-middle Zambezi Rift continues to the south-west into Botswana. Focal depths range up to 50 km (refs 17 and 18).

Westward extension

West of the Okavango the conductive zone runs a few degrees north of due west to the exposed Damara Geosyncline of South-west Africa (Fig. 1), where it cuts obliquely across the WSW-ESE grain^{1,19} of metamorphosed rocks of the eugeosynclinal Swakop Facies of Upper Proterozoic-Lower Palaeozoic ages¹⁸. To the north of the Swakop Facies, the miogeosynclinal Outjo Facies has E-W strikes in its eastern exposures. The conductor runs roughly parallel to the folding in eastern outcrops of Outjo rocks, and to the boundary between the eugeosyncline and the miogeosyncline. It also parallels the northern limit of Damara regional metamorphism¹⁸. No recent major faults are known to run parallel to it. An association with the boundary between two stable crustal blocks seems possible. Several earthquake epicentres lie close to the northern edge of the conductor (Fig. 1).

Origin of conductive zone

Regions of high conductivity which produce geomagnetic induction anomalies may be related to high temperatures in the upper mantle, as in the western USA²⁰, or to compositional conductors such as graphite or saline water which are often concentrated in fracture zones^{21,22}. If the conductive zone under discussion is associated with the African rift system its conductivity could be associated with anomalously high temperature in the upper mantle, or with a fracture zone in the lithosphere, or with both. No decision is possible, but we believe a fracture zone is the likeliest explanation. From Kariba to the Okavango, fractures parallel to the conductor are visible and seismically active (Fig. 1). Seismological evidence for the mantle under the East African rifts shows low P velocities and attenuation of S_n north of a discontinuity near latitude 4°S (refs 13,

15 and 23). For a path Addis Ababa-Nairobi, Rayleigh wave phase velocities are low for a continental path²⁴ and resemble those in the Basin and Range Province of western North America. Elsewhere in Africa phase and group velocities of surface waves and travel times of P_n , S_n and L_3 are typical of stable continental regions^{25,26}. The eastern (Gregory) rift shows a broad negative gravity anomaly north of 4°S and a central positive anomaly, usually interpreted as evidence of intrusion of mantle-derived material, north of 2°S (refs 27-31). There is no central positive anomaly and thus no evidence of a central intrusion under the western rift¹⁵. For no rift is there geophysical evidence of partial melting in the mantle south of 4°S, at which latitude the fracture pattern in the eastern rift changes from grid faulting in the north to block faulting in the south³². Geomagnetic depth sounding across the eastern rift at the Equator³³ reveals anomalies accountable in terms of a conductive region 20 km below the rift floor and another 50 km deep and 100 km east of the rift. Soundings south of 5°S might give different results. Gravity data south of 5°S are sparse and ambiguous and a recent unpublished Bouguer anomaly map of Botswana⁴³ has both highs and lows along the conductor.

The north-east trending arm of the conductor (Fig. 1) can reasonably be associated with the known fractures, whose activity resembles that of the western, Lake Tanganyika rift. The absence of known fractures along the westward arm of the conductor does not preclude their existence because of the Kalahari cover, and seismic activity is present. The westward arm is in line with the Walvis Ridge (Fig. 1), which may, on recent geophysical evidence³⁴, be a fracture zone, either a transform fault or some tectonic process following an older line of weakness; the conductivity anomaly could then lie on a corresponding line of weakness within the continent, along the boundary in pre-Damara basement rocks between the eugeosyncline and the miogeosyncline. In southern Africa pre-existing cratons and orogenic belts have often controlled later tectonics^{4,35-37}. The seismicity may thus trace contemporary failure, under a general WNW-ESE tensile stress field, on pre-existing fractures. The first down-faulting in the middle Zambezi probably occurred during early Mesozoic

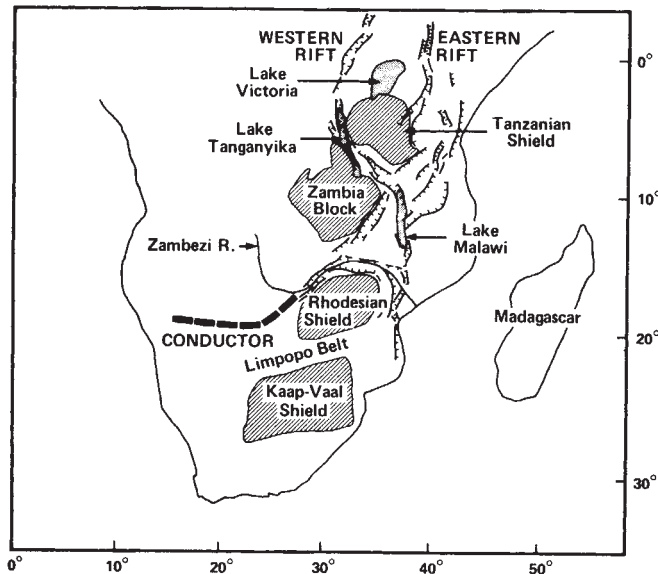


Fig. 2 African rifts and shields in relation to the conductivity anomaly (compiled from a map by McConnell³⁵, and other sources).

times^{4,5} along fractures in the Upper Proterozoic-Lower Palaeozoic mobile zone related to the tectono-thermal Katangan and Damaran Episodes¹⁹. The conductor could mark this zone of weakness in the lithosphere³⁸ along which intermittent movement has occurred since the Upper Proterozoic.

The absence of evidence for opening of rifts in southern Africa can be related to global tectonics in two alternative ways. In terms of classical plate tectonics the pole of opening for the East African rift is in southern Africa^{13,23}, where opening will therefore be small. On the membrane stress hypothesis of Oxburgh and Turcotte³⁹ the rifts are

caused by northward movement of the continent over the equatorial bulge. Again propagating fractures with small displacements would be found in southern Africa.

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- ¹ Haughton, S. H., *Geological History of Southern Africa* (Geological Society of South Africa, Johannesburg, 1969).
- ² Reeves, C. V., *Nature*, **237**, 95-96 (1972).
- ³ Geological Map of Botswana, Scale 1:1000000 (Geological Survey and Mines Department, Lobatse, Botswana, 1973).
- ⁴ Cox, K. G., in *African Magmatism and Tectonics* (edit. by Clifford, T. N., and Gass, I. G.), 211-235 (Oliver and Boyd, Edinburgh, 1970).
- ⁵ Gough, D. I., and Gough, W. I., *Geophys. J. R. astr. Soc.*, **21**, 79-101 (1970).
- ⁶ Vail, J. R., *Geol. Rundsch.*, **57**, 601-614 (1968).
- ⁷ Bailey, D. K., *Geol. Mag.*, **98**, 277-284 (1961).
- ⁸ Lee, C. A., *Geol. Mag.*, **3**, 133-142 (1974).
- ⁹ Gregory, J. W., *The Rift Valleys and Geology of East Africa* (Seeley, London, 1921).
- ¹⁰ Du Toit, A. L., *Geology of South Africa*, third ed. (Oliver and Boyd, Edinburgh, 1954).
- ¹¹ Gane, P. G., and Oliver, H. O., *Trans. geol. Soc. S. Afr.*, **56**, 21-33 (1953).
- ¹² Oliver, H. O., *Trans. geol. Soc. S. Afr.*, **59**, 123-129 (1956).
- ¹³ Fairhead, J. D., and Girdler, R. W., *Geophys. J. R. astr. Soc.*, **21**, 271-301 (1971).
- ¹⁴ Sykes, L. R., *J. geophys. Res.*, **72**, 2131-2153 (1967).
- ¹⁵ Maasha, N., and Molnar, P., *J. geophys. Res.*, **77**, 5731-5743 (1972).
- ¹⁶ Rhodesia Meteorological Services, monthly *Seismological Bulletins*, January to December (1973).
- ¹⁷ Fernandez, L. M., *Seism. Ser. geol. Surv. S. Afr.*, **1**, 1-17 (1972).
- ¹⁸ Fernandez, L. M., and Guzman, J. A., *Seism. Ser. geol. Surv. S. Afr.*, **3**, 1-20 (1973).
- ¹⁹ Clifford, T. N., *Geol. Soc. Am. Spec. Paper* 92 (1967).
- ²⁰ Gough, D. I., *J. Geomag. Geoelectr.*, **26**, 105-123 (1974).
- ²¹ Gough, D. I., and Camfield, P. A., *J. geophys. Res.*, **77**, 3168-3170 (1972).
- ²² Garland, G. D., *Phys. Earth planet. Interiors* (in the press).
- ²³ Baker, B. H., Mohr, P. A., and Williams, L. A. J., *Geol. Soc. Am. Spec. Paper* 136 (1972).
- ²⁴ Knopoff, L., and Schlue, J. W., in *East African Rifts* (edit. by Girdler, R. W.), *Tectonophysics*, **15**, 157-163 (1972).
- ²⁵ Bloch, S., Hales, A. L., and Landisman, M., *Bull. seism. Soc. Am.*, **59**, 1599-1629 (1969).
- ²⁶ Gumper, F., and Pomeroy, P. W., *Bull. seism. Soc. Am.*, **60**, 651-668 (1970).
- ²⁷ Girdler, R. W., and Sowerbutts, W. T. C., *J. Geomag. Geoelectr.*, **22**, 153-163 (1970).
- ²⁸ Darracott, B. W., Fairhead, J. D., and Girdler, R. W., in *East African Rifts* (edit. by Girdler, R. W.), *Tectonophysics*, **15**, 131-141 (1972).
- ²⁹ Searle, R. C., *Geophys. J. R. astr. Soc.*, **21**, 13-31 (1970).
- ³⁰ Baker, B. H., and Wohlenberg, J., *Nature*, **229**, 538-542 (1971).
- ³¹ Girdler, R. W., Fairhead, J. D., Searle, R. C., and Sowerbutts, W. T. C., *Nature*, **224**, 1178-1182 (1970).
- ³² Fairhead, J. D., and Girdler, R. W., in *East African Rifts* (edit. by Girdler, R. W.), *Tectonophysics*, **15**, 115-122 (1972).
- ³³ Banks, R. J., and Ottey, P., *Geophys. J. R. astr. Soc.*, **36**, 321-335 (1974).
- ³⁴ Goslin, J., Mascle, J., Sibuet, J. C., and Hoskins, H., *Bull. Geol. Soc. Am.*, **85**, 619-632 (1974).
- ³⁵ McConnell, R. B., *Bull. Geol. Soc. Am.*, **83**, 2549-2572 (1972).
- ³⁶ Fyfe, W. S., and Leonardos, O. H., *Nature*, **244**, 501-502 (1973).
- ³⁷ Thorpe, R. S., and Smith, K., *Earth planet. Sci. Lett.*, **22**, 91-95 (1974).
- ³⁸ Watterson, J., *Nature*, **253**, 520-521 (1975).
- ³⁹ Oxburgh, E. R., and Turcotte, D. L., *Earth planet. Sci. Lett.*, **22**, 133-140 (1974).
- ⁴⁰ Geological Map of South-West Africa, Scale 1:1000000 (Government Printer, Pretoria, South Africa, 1964).
- ⁴¹ Provisional Geological Map of Rhodesia, Scale 1:1000000 (Rhodesia Geological Survey, Salisbury, Rhodesia, 1971).
- ⁴² International Tectonic Map of Africa, Scale 1:5000000 (Unesco, 1968).
- ⁴³ Reeves, C. V., and Hutchins, D. G., *Nature*, **254**, 408-410 (1975).

New method for mapping genes in human chromosomes

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If human cells are subjected to large doses of ionising radiation and then fused with rodent cells, hybrid clones are obtained in which linked human genes may be segregated. By measuring the frequency with which pairs of linked genes are cotransferred after irradiation, it is possible to determine the linear order of groups of genes and to estimate the distances between them. This technique offers a new and systematic approach to the mapping problem.

RECENT progress in the assignment of genes to specific human chromosomes has been made possible by four developments: first, the provision of a general method for fusing together cells from different animal species and the demonstration that such fused cells can give rise to mononucleate hybrids capable of

indefinite multiplication^{1,2}; second, the application of selective media to facilitate the isolation of the hybrid cells³; third, the discovery that in hybrids produced by fusing human cells with those of rodents most of the human chromosomes are rapidly and preferentially eliminated⁴; and finally, the detection, in appropriately stained preparations, of specific banding patterns in the chromosomes that permit their accurate identification⁵. By means of experiments in which the retention of human biochemical markers in man-mouse (or man-hamster) hybrid cells is correlated with the retention of identifiable human chromosomes, some fifty human genes have been reliably assigned to specific chromosomes⁶. The success of these experiments does not, however, alter the fact that, compared with what can be achieved in prokaryotic systems, this form of linkage analysis, limited in whole chromosomes, is a very crude form of mapping. Some modest success in determining the position

of genes within the chromosome has been achieved by exploiting translocations that segregate linked markers⁷⁻⁹. But this approach is unsystematic in that it is constrained by the availability of informative translocations involving the particular chromosome being studied, and limited to resolution in that genes can only be localised to a segment of chromosome large enough to be identified in a translocation. For mammalian cells, a general method for mapping the order and spacing of linked genes is still lacking.

In this paper we present a new approach to the mapping problem and illustrate its efficacy in determining the position of four genes on the long arm of the X chromosome in man. The principle underlying this approach was suggested by two sets of observations. First, chromosomes may be fragmented and parts of chromosomes translocated by ionising radiation; the frequency of chromosomal rearrangements is reproducibly determined by the dose of radiation administered¹⁰. It will be obvious that when chromosomes are fragmented, the probability of two linked genes being included within a single fragment will be inversely related to the distance between them. Second, fragments of foreign genetic material can, in certain conditions, be introduced into mammalian cells in such a way that they are replicated and expressed even though they may be too small to be detectable in cytological preparations¹¹⁻¹⁴.

We therefore subjected human cells to controlled doses of radiation, fused them with unirradiated hamster cells and selected clones of hybrid cells expressing a marker known to be coded by a gene located on the long arm of the human X chromosome. We then measured the frequency with which three other genes known to be located on this arm of the X chromosome were cotransferred with the selected marker. From these measurements we were able to determine the order of the four genes and estimate the relative distances between them.

Selection of hybrid clones

Human diploid male lymphocytes, purified from venous blood by the Ficoll-Isopaque technique¹⁵, were irradiated and fused immediately after irradiation with the hamster cells. The lymphocytes, which had not been subjected to any specific mitogenic stimulation, were thus very largely a non-dividing cell population; each cell contained only one copy of the X chromosome. The same lymphocyte donor was used for all experiments to reduce as far as possible variation between different batches of cells. The conditions of irradiation were made as reproducible as possible: the equipment embodied a double caesium source which delivered gamma rays at a constant dose of 120 rad min⁻¹. During irradiation cells were suspended in ice-cold Eagle's MEM without serum. The radiation dose was determined by the duration of exposure to the caesium source. Since radiation damage seems not to be repaired at low temperatures^{16,17}, we assume that in these conditions the total radiation dose administered accurately reflects the total damage sustained by the cells.

The hamster line used was Wg3-H, a DON line derivative lacking hypoxanthine guanine phosphoribosyl transferase (HGRT) activity¹⁸. This cell line generates wild type revertants at a very low frequency, but it was, in any case, propagated in medium containing 6 µg ml⁻¹ of thioguanine, which is lethal to wild type cells. Wg3-H cells (3×10^6) were fused with 10×10^6 – 12×10^6 irradiated lymphocytes by the technique described by Harris and Watkins¹. Haemagglutinating units (5,000) of inactivated Sendai virus were added to the mixed cell suspensions to make a total volume of 0.5 ml. After fusion, the cells were distributed into 20 small Falcon flasks in MEM. The medium was replaced the following day by HAT medium, which is MEM containing 6×10^{-5} M hypoxanthine, 10^{-5} M thymidine, 4.5×10^{-7} M aminopterin and 4×10^{-4} M glycine. During the medium change, unfused lymphocytes are washed away; unfused hamster cells or fused hamster cells that had not received a lymphocyte component remain deficient in HGRT

Table 1 The frequency of cotransference of HGRT with other X-linked markers

Dose krad	Observed frequency of co-transference of HGRT with the unselected markers:			No. of clones examined
	PGK	α -gal	G6PD	
0	0.95	0.95	0.95	46
1	0.80	0.86	0.90	51
2	0.55	0.68	0.75	78
4	0.22	0.34	0.42	77

and are killed in HAT medium; only man-hamster hybrid cells proliferate. No colonies developed in control flasks seeded with hamster cells that had been treated with Sendai virus in the absence of added human lymphocytes. Only one colony was isolated from each experimental flask to ensure that all clones studied were of independent origin.

Species specificity of markers

The four X-linked markers studied were the enzymes HGRT, phosphoglycerate kinase (PGK), glucose-6-phosphate dehydrogenase (G6PD) and α -galactosidase (α -gal). The human forms of all these enzymes can be separated from the hamster forms by electrophoretic techniques. The human forms of HGRT, PGK and G6PD were identified by Cellogel electrophoresis essentially as described by Meera-Khan¹⁹. The human and hamster forms of α -gal, as detected by hydrolysis of the fluorogenic substrate 4-methyl-umbelliferyl- α -D-galactopyranoside²⁰, can also be separated by Cellogel electrophoresis in the same buffer as that used for HGRT. Separation of the two forms of the enzyme is simplified if the preparations are treated with neuraminidase²¹. Before electrophoresis, the extracts were therefore incubated at 37 °C for 45 min with an equal volume of neuraminidase from *V. cholerae* (BDH) (500 units ml⁻¹).

Determination of gene order

All clones isolated were assumed to contain the human gene for HGRT. In early experiments, each clone was actually tested for the presence of the human enzyme, but since no revertants containing the hamster enzyme were found, the presence of other human X-linked enzymes was accepted as evidence of the hybrid nature of the clone. The species of origin of the HGRT was checked in all clones that showed no other human enzymes. Table 1 lists the frequencies with which the unselected human markers PGK, G6PD and α -gal were cotransferred with HGRT in the hybrid clones. It is clear that the frequency of cotransference of these markers is reduced as the dose of irradiation administered to the human cells is increased. If the human cells are not irradiated, segregation of these markers from HGRT occurs very rarely, and a cytologically identifiable human X chromosome can usually be seen in the hybrid cells. It is very unlikely that direct inactivation of the structural genes by gamma rays, or of other genes governing expression of the markers, contributes significantly to the observed frequencies of segregation; direct inactivation of genes would be expected to occur at a very much lower frequency²². The most probable explanation for the segregation of the unselected human markers from HGRT is that the genes coding for these markers are separated from that coding for HGRT by radiation-induced chromosome breaks and translocations. Once separated from HGRT, which is maintained in the hybrid cell populations by selective pressure, chromosome fragments bearing unselected markers would be rapidly lost. If this explanation is, in principle, correct, then the fact that PGK segregates from HGRT more frequently than either α -gal or G6PD indicates that PGK is further removed from HGRT than are the other two markers.

The problem is susceptible to quantitative treatment. We define a segregational event as any event that disrupts the

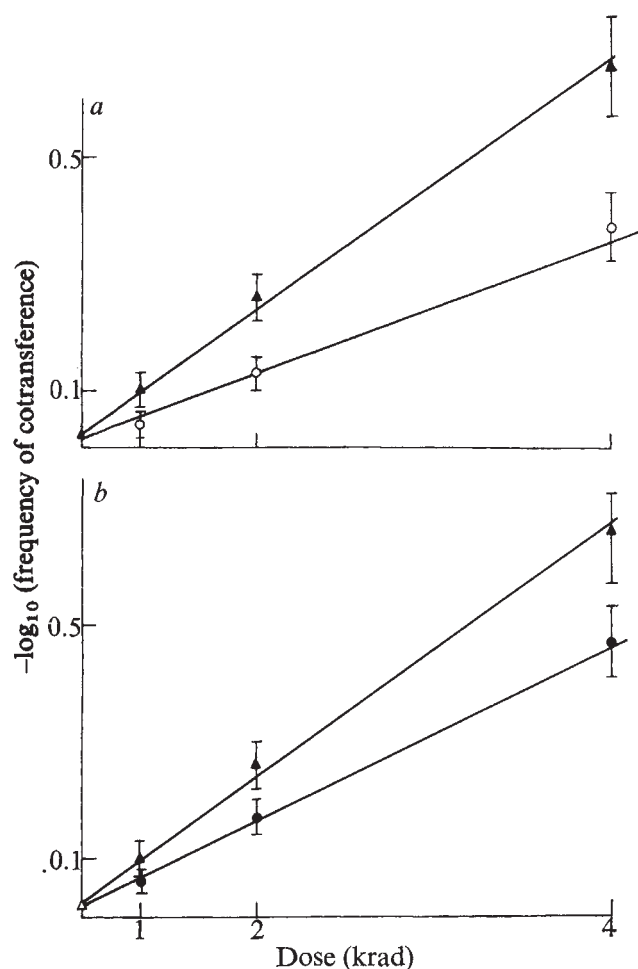


Fig. 1 The logarithms of the frequencies of cotransference of PGK with HGRT, G6PD with HGRT and α -gal with HGRT are plotted against the dose of radiation. The dose axis has been expanded as $(\text{Dose})^{1.6}$, an empirically determined function which converts the plots to linearity. The slopes of the lines are proportional to the targets for the segregation of each pair of loci (see text). The target PGK/HGRT is compared with G6PD/HGRT in (a), and with α -gal/HGRT in (b).

The target sizes calculated relative to PGK/HGRT are: PGK/HGRT = 10 units (arbitrary standard); G6PD/HGRT = 5.4 ± 0.9 ; α -gal/HGRT = 6.9 ± 1 . (Margins of ± 1 s.e. are used throughout.) $\blacktriangle$: PGK/HGRT; $\circ$: G6PD/HGRT; $\bullet$: α -gal/HGRT.

linkage of two genetic loci. The number, S , of such events induced in the whole genome by irradiation is obviously determined by some function of the dose of radiation delivered. We may therefore write:

$$S = f(D)$$

Events that segregate one locus from another may be considered as occurring within a specific target delimited by the two loci, this target being equivalent to some fraction t of the whole genome. If we assume that, to a first approximation, segregational events induced in interphase chromatin are randomly distributed throughout the whole genome, then the mean number of events $\bar{S}_t$ occurring in target t is given by:

$$\bar{S}_t = t.f(D)$$

The actual numbers of such events occurring in this target will be scattered around the mean value $\bar{S}_t$ with the frequency distribution described by Poisson's theorem. We can therefore use this theorem to predict the probability (P_0) that the number

of such events will be zero, that is, the probability that the linkage will not be disrupted:

$$P_0 = \exp[-t.f(D)]$$

If the viability of the hybrid cell is not significantly affected by the segregation of any particular unselected marker, then P_0 can be equated with the frequency F of occurrence of hybrid clones in which the two loci are cotransferred.

Then

$$F = \exp[-t.f(D)]$$

and

$$-\log F = t.f(D)$$

This relationship predicts that if the logarithm of the frequency of clones in which two loci are cotransferred is plotted against an appropriate function of the radiation dose, a straight line should be defined with a slope proportional to the size of the target for the segregation of the two loci. Fig. 1 shows that this prediction seems to be correct. The appropriate function of dose was determined empirically to be approximately $D^{1.6}$ and, within the limits of experimental error, was found to be the same for any pair of loci. A dose exponent between 1 and 2 has commonly been found for the frequency of chromosome rearrangements induced by X or gamma rays¹⁰. This exponent indicates that such rearrangements involve a combination of single and double hit events. Calculation of target size does not, however, require an accurate estimate of the dose function. Provided that this function is the same for all pairs of loci, relative target sizes can be calculated from the ratios of the logarithms of the frequencies of the non-segregant clones. In Fig. 1 the data are plotted for the cotransference of PGK with HGRT (target PGK/HGRT), of α -gal with HGRT (target α -gal/HGRT) and of G6PD with HGRT (target G6PD/HGRT). The target sizes are calculated relative to the target PGK/HGRT which is arbitrarily assigned a value of 10 units.

If, as we suppose, segregational events are largely determined by chromosomal rearrangements, then one would expect that two unselected markers situated on opposite sides of a selected marker would be segregated from that marker in an independent fashion. Thus, if G6PD and PGK were situated on opposite sides of HGRT, then the segregation of either one of these markers from HGRT should be independent of the segregation of the other. In Table 2, the observed numbers of clones expressing both the markers PGK and G6PD (P^+G^+), neither marker (P^-G^-) or either marker (P^+G^- or P^-G^+) are compared in each case with the numbers expected if PGK and G6PD segregated from HGRT independently. It will be seen that the observed numbers are not significantly different from

Table 2 A test of the independence of the segregation of PGK and of G6PD from HGRT

Class of segregant	Number of hybrid clones in each class at each dose			Total
	1 krad	2 krad	4 krad	
P^+G^+ expected	37	31.6	7.1	75.7
observed	38	34	10	82
P^-G^- expected	1.0	8.6	35.1	44.7
observed	2	11	38	51
P^+G^- expected	4.0	10.4	9.9	24.3
observed	3	8	7	18
P^-G^+ expected	9.0	26.4	24.9	60.3
observed	8	24	22	54

The number of clones observed in each of the possible classes of segregant for the two markers PGK and G6PD is compared with that expected* if PGK and G6PD segregated independently from HGRT. $\chi^2 = 3.7$ (calculated from the totals, with 1 d.f.).

*If the overall frequency, at any one dose, for the segregation of PGK is p^- and of G6PD is g^- , the number of clones expected in each class, when a total of n clones is examined, will be: for P^-G^- , $n.p^-g^-$; for P^+G^- , $n.(1-p^-)g^-$; for P^-G^+ , $n.p^-(1-g^-)$; for P^+G^+ , $n.(1-p^-)(1-g^-)$.

the expected numbers ($\chi^2 = 3.7$). We may therefore conclude that the segregation of G6PD from HGRT is essentially independent of the segregation of PGK from HGRT and thus that G6PD and PGK must be on opposite sides of HGRT. Although the differences between the observed and the expected numbers are not statistically significant, there does seem to be some indication that the segregation of the two markers from HGRT is not totally independent, for the observed numbers of P^+G^- and P^-G^+ clones are consistently higher than expected, and the observed numbers of P^+G^+ and P^-G^- clones are consistently lower. A possible explanation for this marginal effect is discussed later.

A similar analysis of the segregation of α -gal and G6PD from HGRT shows that these two loci must also be situated on opposite sides of HGRT. It then follows that α -gal and PGK must lie on the same side of HGRT and, since the target α -gal/HGRT (6.9) is smaller than the target PGK/HGRT (10), that α -gal must be situated between HGRT and PGK. If this is so, then the segregation of PGK from HGRT must be dependent in part on the segregation of α -gal, for any segregational event occurring between α -gal and HGRT would also segregate PGK from HGRT. Table 3, which is analogous to Table 2, shows that this is indeed the case: the concordant segregants ($P^+\alpha^+$ and $P^-\alpha^-$) occur much more frequently, and the independent segregants $P^+\alpha^-$ and $P^-\alpha^+$ occur much less frequently than would be expected if PGK and α -gal segregated from HGRT independently. In this case, the differences between the numbers of clones observed in each class and the numbers expected are highly significant ($\chi^2 = 36.4$). Our model is thus substantiated and we can confidently deduce from our data that the order of the four genes is:

PGK α -gal HGRT G6PD

This gene order is in accordance with that derived from the consensus of studies involving the use of spontaneous translocations⁹.

Target size and map distance

Although the target size for the segregation of two loci is a measure of the map distance between the loci, we cannot assume that the target size is directly proportional to the map distance. This would be so if the only type of event causing the segregation of loci was a chromosome break (or translocation) that separated all the loci on one side of the break from all the loci on the other. One locus could be segregated, however, from another by a locally acting event, for example by direct inactivation of the gene, and the frequency of such an event would clearly be independent of the distance between the two loci. We have already noted that the expected frequency of gene mutation (of the order 10^{-3}) is too low to have any appreciable influence on our results; but it is apparent from our data that a genetic locus can be segregated by a local event of some other kind. For example, Table 3 shows a few cases in which α -gal has been lost while PGK and HGRT are retained, an event formally equivalent to an interstitial deletion. Events of this kind would contribute to our estimates of the target size for the segregation of two loci an increment independent of the map distance between them. We can calculate the effective target size for one such event by scoring the frequency with which α -gal is retained in clones expressing both PGK and HGRT: the calculations yield a target size of 1.7 ± 0.6 units.

There is no reason to suppose that localised segregational events of this kind are limited to the α -gal locus; but with our present data we can attempt to demonstrate their occurrence at only one other locus, HGRT. If the HGRT locus could be separated from the rest of the chromosome by a localised event and incorporated alone in a hybrid cell, then that cell would be scored as one in which all the other three loci had segregated. Thus, occasionally, a single event would simultaneously segregate loci on opposite sides of the selected marker. This effect

might explain why, in Table 2, there is some suggestion that the segregation of PGK from HGRT may not be totally independent of the segregation of G6PD from HGRT. The target for such a localised event at the HGRT locus would thus contribute to the targets for the segregation of any other marker from HGRT. This contribution is easily eliminated from the target for the segregation of α -gal from HGRT by recalculating this target from the data on clones in which a localised segregational event at HGRT cannot have occurred, for example, on clones that express G6PD. This new target, α -gal/HGRT(G^+), would still include localised segregational events at α -gal, but we have already calculated the target for these (1.7 ± 0.6), so that the true map distance between α -gal and HGRT is obtained by subtracting 1.7 from the target α -gal/HGRT(G^+). The target α -gal/HGRT(G^+) and the analogous targets PGK/HGRT(G^+) and G6PD/HGRT(P^+ or α^+) are then as follows: α -gal/HGRT(G^+) 5.6 ± 1 ; PGK/HGRT(G^+) 8.3 ± 1.4 ; G6PD/HGRT(P^+ or α^+) 4.1 ± 0.9 . These figures are less than the targets given in Fig. 1, which include the effects of localised segregational events at HGRT, by 1.3, 1.7, and 1.3 respectively. Although these differences, which estimate the target for such events at HGRT, only approach statistical significance with the data we have so far obtained, it seems none the less that this target is similar in size to the target for localised segregational events at α -gal. It seems reasonable to assume that similar targets exist at the PGK and the G6PD loci, but further work is needed to demonstrate this. On this assumption, a provisional statement of map distances would then be: PGK-HGRT: 6.6 ± 1.5 ; PGK- α -gal: 3.3 ± 1.2 ; α -gal-HGRT: 3.9 ± 1.2 ; HGRT-G6PD: 2.4 ± 1.1 .

Systematic approach to mapping

The investigations we have described show that the technique of cell fusion coupled with quantitative irradiation of one of the parent cells provides a systematic approach to the mapping of genes within mammalian chromosomes. This approach rests heavily on the presence in a particular chromosome of a selectable marker, although, much more laboriously, it could be applied without such selection. However, since selective procedures exist for genes on chromosomes other than the X and since several X-autosome translocations are available, the technique, without substantial modification, is immediately applicable to a large part of the human genome. A modest number of hybrid clones will reveal the order of the genes, and the accuracy of determination of target size, and hence map distance, is limited only by the number of clones analysed. Karyological analysis is not necessary (except where orientation with respect to the centromere is required) and is thus not a limiting factor in the resolution of the analysis. Since scoring is based on the frequency of non-segregant clones, all clones are informative with respect to all loci studied.

Table 3 To show that PGK and α -gal do not segregate independently from HGRT

Class of segregant	Number of hybrid clones in each class at each dose			Total
	1 krad	2 krad	4 krad	
$P^+\alpha^+$ expected	35.4	29.2	5.7	70.3
observed	40	39	12	91
$P^-\alpha^-$ expected	1.4	11.2	39.7	52.3
observed	6	21	46	73
$P^+\alpha^-$ expected	5.6	13.8	11.3	30.7
observed	1	4	5	10
$P^-\alpha^+$ expected	8.6	23.8	20.3	52.7
observed	4	14	14	32

The number of clones observed in each of the possible classes of segregant for the two markers PGK and α -gal is compared with that expected if PGK and α -gal segregated independently from HGRT. $\chi^2 = 36.4$ (calculated from the totals, with 1 d.f.).

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- ¹ Harris, H., and Watkins, J. F., *Nature*, **205**, 640-646 (1965).
- ² Yerganian, G., and Nell, N. B., *Proc. natn. Acad. Sci. U.S.A.*, **55**, 1066-1073 (1966).
- ³ Littlefield, J. W., *Science*, **145**, 709-710 (1964).
- ⁴ Weiss, M. C., and Green, H., *Proc. natn. Acad. Sci. U.S.A.*, **58**, 1104-1111 (1967).
- ⁵ Caspersson, T., Modest, E. J., Foley, G. E., Wagh, U., and Simonsson, E., *Expl Cell Res.*, **58**, 128-140 (1969).
- ⁶ Ruddle, F. H., *Nature*, **242**, 165-169 (1973).
- ⁷ Miller, D. A., and Miller, O. J., *Science*, **178**, 949-955 (1972).

- ⁸ Boone, C., Chen, T., and Ruddle, F. H., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 510-514 (1972).
- ⁹ Gerald, P. S., and Brown, J. A., *Cytogenet. Cell Genet.*, **13**, 29-34 (1974).
- ¹⁰ Evans, H. J., pp 191-237 in *Chromosomes and Cancer* (edit. by German, J.) (Wiley, New York, 1974).
- ¹¹ Schwartz, A. G., Cook, P. R., and Harris, H., *Nature new Biol.*, **230**, 5-8 (1971).
- ¹² Boyd, Y. L., and Harris, H., *J. Cell Sci.*, **13**, 814-861 (1973).
- ¹³ Klinger, H. P., and Shin, S., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1398-1402 (1974).
- ¹⁴ McBride, W. O., and Ozer, H. L., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1258-1262 (1973).
- ¹⁵ Böyum, A., *Scand. J. clin. Lab. Invest. Suppl.* **97**, 77-89 (1968).
- ¹⁶ Kleijer, W. J., Hoeksema, J. L., Sluyter, M. L., and Bootsma, D., *Mutat. Res.*, **17**, 385-394 (1973).
- ¹⁷ Dewey, W. C., Miller, H. H., and Leeper, D. B., *Proc. natn. Acad. Sci. U.S.*, **68**, 667-671 (1971).
- ¹⁸ Westerveld, A., Visser, R. P. L. S., Meera Khan, P., and Bootsma, D., *Nature new Biol.*, **234**, 20-24 (1971).
- ¹⁹ Meera Khan, P., *Archs Biochem. Biophys.*, **145**, 470-483 (1971).
- ²⁰ Grzeschik, K. H., et al., *Nature new Biol.*, **240**, 48-50 (1972).
- ²¹ Meera Khan, P., Westerveld, A., Würzer-Figurelli, E. M., and Bootsma, D., *Proc. second int. Conf. Human Gene Mapping*, (in the press).
- ²² Albertini, R. J., and DeMars, R., *Mutat. Res.*, **18**, 199-224 (1973).

Cholesterol is excluded from the phospholipid annulus surrounding an active calcium transport protein

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When phospholipids in the first shell of the lipid bilayer surrounding a calcium transport protein are replaced by cholesterol, there is a complete and reversible inactivation of ATPase activity. This phospholipid annulus normally excludes cholesterol and the lateral segregation of the lipids is essential for the protein to function.

LIPIDS and proteins can diffuse rapidly in the plane of some membranes at rates of up to several microns per second^{1,2}. This fluidity prompts the question of whether persistent interactions can occur between specific proteins and lipids in the bilayer, which normally contains a very heterogeneous mixture of chemically distinct lipids. The question may be acute for a protein that is inserted into a lipid bilayer containing substantial proportions of lipid which will not support its function. To retain function the tertiary structure of the protein must be able to specify an appropriate lipid environment.

Cholesterol is a common component of membrane lipids, and we have found that when it is forced to interact directly with a number of membrane proteins it has a strong inhibitory effect on their function. The structure of the membrane is designed to exclude direct interaction of cholesterol with the protein. This exclusion is caused by a single shell of phospholipid bilayer bound relatively tightly to the protein, which we term the annulus. We are therefore suggesting that the protein imposes a lateral segregation of lipid classes which ensures that the protein is maintained in a functional conformation.

Evidence for the exclusion of cholesterol from the phospholipid annulus is derived primarily from the ATPase which is responsible for the active transport of calcium into sarcoplasmic reticulum (SR). Two calcium ions are taken up into purified SR vesicles for each ATP molecule hydrolysed³, and 80% of the membrane protein is the ATPase responsible for calcium uptake⁴. Biochemical and spin label evidence led us to propose a structural model in which about 30 phospholipid molecules

interact directly with the hydrophobic surface of the ATPase spanning the bilayer, and form the minimal lipid environment required to maintain full ATPase activity of the protein^{5,6}. (See also the model proposed previously for cytochrome oxidase complexes with lipid¹².)

The 30 lipid molecules in the annulus can be replaced almost entirely with defined lipids by the technique of lipid substitution⁴⁻⁷. All the essential structural features of the protein are conserved, as the pure ATPase-lipid complexes can be incorporated into vesicles which actively accumulate calcium⁴. We have adapted the lipid substitution technique to prepare ATPase-lipid complexes which contain cholesterol. Comparison of the properties of the various complexes provides evidence for the exclusion of cholesterol from the phospholipid annulus.

Cholesterol does not affect activity of ATPase with a complete phospholipid annulus

When phospholipids are added to complexes of pure ATPase with a complete annulus of about 30 SR phospholipids (termed SR-ATPase), there is a rapid and complete equilibration of the phospholipid pools in the presence of appropriate concentrations of cholate⁶. The activity of the ATPase is then determined by the composition of the phospholipids in the annulus: for example, dimyristoyl lecithin (DML) and dipalmitoyl lecithin (DPL) inhibit the activity of pure SR-ATPase complexes to about the same extent, and the inhibition is a linear function of the lipid composition (Fig. 1). In contrast, if SR-ATPase is titrated in the presence of cholate with an excess of dioleoyl lecithin (DOL), DML or DPL, containing increasing proportions of cholesterol, the activity of the equilibrated mixtures is virtually unaffected by the presence of cholesterol at up to equimolar proportions with the added lecithins (Fig. 2). If a cholate titration complex of SR-ATPase mixed with equimolar DOL-cholesterol is centrifuged into a sucrose gradient an ATPase-lipid complex is recovered in which the proportion

of DOL in the annulus corresponds to at least 90% of the expected substitution of the added DOL for the SR lipid (Table 1). The presence of cholesterol does not therefore prevent the normal equilibration of phospholipids which is facilitated by cholate.

These results are most simply interpreted by assuming that cholesterol does not significantly affect the interaction of the phospholipids with the ATPase, and that the phospholipids exert the same effect on the activity of the enzyme irrespective of the presence of cholesterol. It is possible that a normal interaction of cholesterol with the enzyme is prevented in some way by the use of cholate as the equilibrating agent, perhaps because of the structural similarity between this detergent and cholesterol. This possibility is ruled out by experiments in which cholesterol was introduced into ATPase-lipid complexes by cosonication of SR-ATPase with increasing amounts of equimolar DOL and cholesterol. The resulting ATPase-lipid

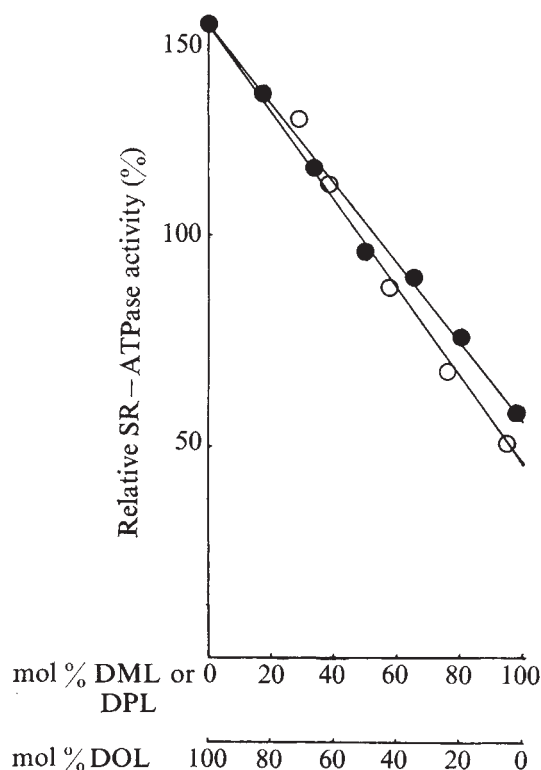


Fig. 1 Titrations of ATPase activity with defined lecithins. SR-ATPase (0.20 mg) was treated with a total of 1 mg phospholipid (DOL-DML or DOL-DPL) and 0.5 mg cholate in 100 μ l sucrose buffer containing 250 mM sucrose, 1 M KCl, 5 mM $MgATP^{2-}$, and 50 mM potassium phosphate, pH 8.0. After incubation at 40 °C for 10 min, 5 μ l samples were diluted 200-fold into ATPase assay medium at 37 °C. Added phospholipids comprised 95% of total phospholipid pool and the 100% SR-ATPase activity was 12.0 IU mg^{-1} . Activity of different pure SR-ATPase preparations varied between 12–14 IU mg^{-1} at 37 °C. ●, DML; ○, DPL.

complexes were collected by centrifugation into a sucrose gradient to separate them from excess lipid which had not become incorporated by sonication. Table 2 shows that a small proportion of the added DOL-cholesterol is fused with the SR-ATPase complexes, and that there is a small increase in the ATPase activity with increasing proportions of DOL-cholesterol in the complexes, similar to that observed by cosonication with a large excess of DOL alone. Note that the activation of the SR-ATPase by sonication with a 20-fold excess of DOL is similar to that obtained by equilibration of DOL with SR-ATPase by cholate (30% compared with 38% in Fig. 2). To show that the cosonication procedure caused fusion, SR-ATPase was cosonicated with DML or equimolar DML-cholesterol. In both experiments the ATPase activity was

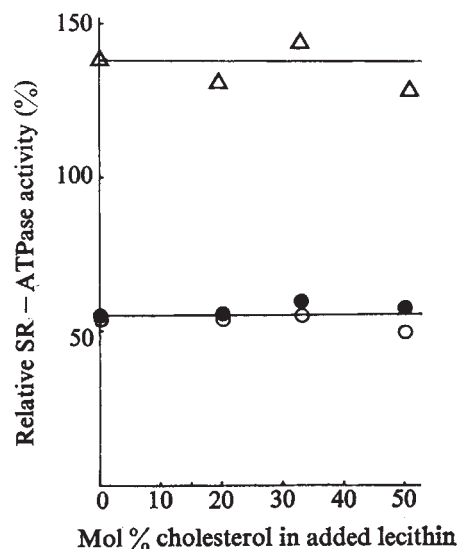


Fig. 2 Titration of ATPase activity with defined lecithin-cholesterol mixtures. SR-ATPase (0.25 mg) was treated with 0.5 mg phospholipid (Δ , DOL; ●, DML or ○, DPL) containing 0–50 mol per cent cholesterol, and 0.25 mg cholate in 100 μ l sucrose buffer. Mixture was incubated at 40 °C and assayed at 37 °C. Added phospholipid comprised 90% of total phospholipid pool.

reversibly inhibited, indicating a direct interaction of the DML molecules with the enzyme. The inhibition was less than in the presence of cholate because the fusion caused by sonication is incomplete (see ref. 6 for examples of similar experiments). These experiments confirm that cholesterol has no effect on ATPase activity in conditions in which mixing of the phospholipid pools interacting directly with the ATPase can be clearly demonstrated.

As cholesterol replaces phospholipids in the annulus ATPase activity is progressively inhibited

To force cholesterol to interact directly with the ATPase, it is necessary to use high concentrations of cholate which strip phospholipids from the annulus^{5,6}. If this stripping is performed in the presence of cholesterol, it substitutes for the displaced phospholipids and the enzyme is maintained in a stable but inactive conformation. These complexes can only be reactivated fully by displacing the cholesterol from the annulus by phospholipid.

Fig. 3 shows the relative ATPase activities of 20 separate preparations of ATPase-lipid complexes prepared by treating

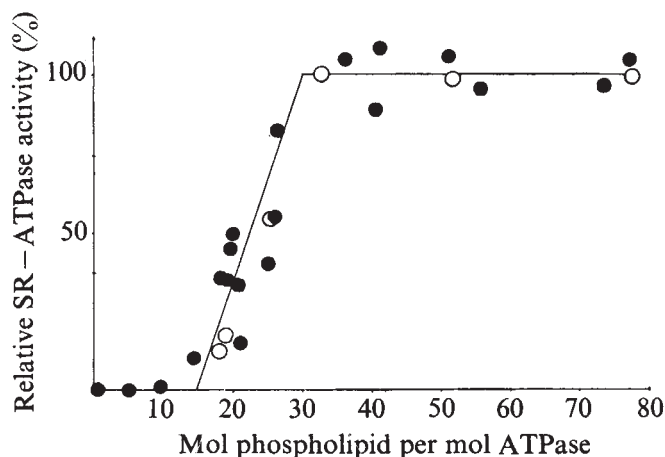


Fig. 3 Effect of replacing phospholipids in annulus by cholesterol. SR vesicles (15 mg) were treated with 0–40 mg cholesterol in cholate at 0.5 mol cholate per mol cholesterol in a total volume of 1.5 ml sucrose buffer. Incubation, isolation and analysis of the complexes was performed as in the legend to Table 1. Cholesterol content of complexes varied between 10–95 mol %. ○, experiments performed in absence of cholesterol.

Table 1 Substitution of phospholipid annulus by DOL in the presence of cholesterol

SR treated with:	ATPase activity (IU mg ⁻¹) at 37 °C	Lipid composition of complexes (mol per mol ATPase)			% Substitution of SR phospholipids by DOL	
		Cholesterol	SR lipid	DOL	Expected*	Observed
DOL	13.8	—	3.2	31.5	92	91
DOL + cholesterol	12.0	24.0	5.4	28.9	92	84

*For complete equilibration of added DOL and SR lipid pools.

SR-ATPase (8.5 mg) was treated with 20 mg DOL, 10 mg cholate ± 10 mg cholesterol in a total volume of 1.5 ml sucrose buffer. Mixture incubated at 0 °C for 30 min and layered on 2 ml 20% (w/v) sucrose, 0.2 ml 50% sucrose and 0.2 ml 80% sucrose in 1 M KCl and 50 mM potassium phosphate, pH 8.0. After centrifugation for 15 h at 5 °C and 200,000g_{av}, the supernatant was discarded and the pellet washed with sucrose buffer until the level of cholate had fallen below 0.5 mol cholate per mol ATPase. Lipids were analysed using radiolabelled DOL and cholesterol, and SR lipids by gas liquid chromatography.

SR with cholesterol in the presence of cholate at various concentrations. The complexes were isolated by centrifugation into a sucrose gradient and the residual cholate level reduced to less than 0.5 molecules per ATPase by dialysis. Note that the lipid composition of the isolated complexes depends on the temperature and time of incubation as well as the concentration of cholate, lipid, and ATPase protein (see legend to Fig. 3). Fig. 3 shows that the activity of the complexes is maximal and unaffected by the presence of cholesterol until there are less than about 30 phospholipid molecules per ATPase in the complexes isolated by centrifugation. In all these complexes, the proportion of cholesterol in the lipid varied from 10–95 mol %. Below 30 phospholipid molecules per ATPase there is a sharp drop in ATPase activity, until below about 15 phospholipids per ATPase the complexes have negligible activity. This activity profile is very similar to that observed by increasing the cholate concentration in the absence of cholesterol, also shown in Fig. 3. The important difference is that the complexes inactivated by cholesterol can be reactivated by adding back phospholipid (SR or DOL) in the presence of cholate. Several reactivation experiments are shown in Fig. 4, in which the ATPase-lipid complexes have been titrated with either the precise amount of phospholipid required to complete an annulus of 30 phospholipid molecules, or a much larger excess of phospholipid (about 500–1,000 molecules per ATPase) to achieve maximal reactivation. The partially inactivated complexes can be fully reactivated provided they contain a total of at least 25–30 phospholipid plus cholesterol molecules per ATPase. Complexes with a lower lipid complement prepared by using very high cholate concentrations can only be partially reactivated (Fig. 4). For example, one very inactive complex, containing only 0.7 mol SR phospholipid and 12 mol cholesterol was

reactivated 380-fold by a large excess of phospholipid, to 10% of the maximal ATPase activity. Figure 4 also shows that a stoichiometric addition of phospholipid calculated to give a total of 30 phospholipids per ATPase restores 75–80% of the maximal activity of the complex. Furthermore these stoichiometric complexes show a further slow increase in activity over a period of 10–20 h after the initial reactivation.

These experiments show that cholesterol causes a potent inhibition of activity when it replaces phospholipids in the annulus, and that equimolar cholesterol and phospholipid in annulus completely and reversibly inhibits the ATPase. Most of the inhibition caused by cholesterol is abolished by sufficient phospholipid to reform a complete annulus.

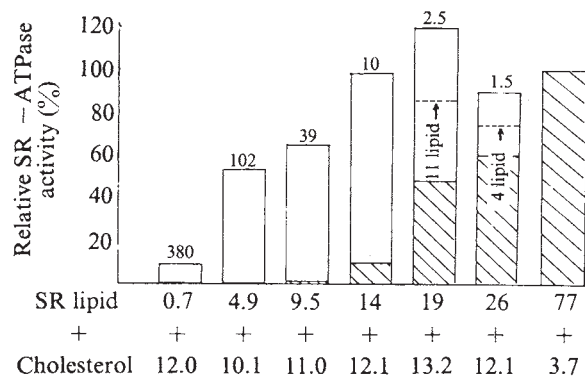
Functional significance of phospholipid annulus

The absence of a significant effect of cholesterol on ATPase activity (< ±10%) when there is a complete phospholipid annulus, implies that not more than two of the lipids in the annulus can be cholesterol molecules even when cholesterol and phospholipid are present in equimolar proportions in the membrane. It is clearly essential for the function of the ATPase that it should be shielded from the strong inhibitory action of cholesterol by an appropriate lateral arrangement of the lipids in the bilayer. The similarity of these ATPase results with preliminary data for three mitochondrial enzymes which have been titrated with cholesterol in the presence of high cholate concentrations, suggests that a major class of membrane proteins with diverse biochemical functions will be found to exclude cholesterol from their immediate environment. For example, when mitochondria from an ox heart were titrated with an excess of equimolar mitochondrial lipid and cholesterol, the

Table 2 Effect of cosonicated SR-ATPase with DOL-cholesterol mixtures

Molar ratio of DOL-cholesterol to endogenous SR lipid in cosonicated mixture	Lipid composition of the isolated complexes (mol per mol ATPase)			% Relative activity at 37 °C
	Endogenous	DOL	Cholesterol (mol%)	
0:1	29	—	2.5 (7.5)	100
1:1	29	1.9	3.0 (8.8)	98
2:1	24	5.7	7.6 (20)	114
5:1	29	14	17 (28)	110
10:1	28	29	35 (38)	121
20:1	24	69	78 (46)	128
20:1, DOL: SR lipid	26	80	3.0 (2.8)	130

SR-ATPase (7.5 mg) was treated with 0–25 mg equimolar sonicated DOL and cholesterol in a total volume of 1.5 ml 50 mM sucrose buffer. Mixture sonicated under N₂ for 5 min at 20–25 °C and layered on 2 ml 5% (w/v) sucrose and 0.2 ml 50% sucrose in 1 M KCl, 50 mM potassium phosphates, pH 8.0. After centrifugation at 5 °C for 1 h at 200,000g_{av}, the pellets were collected and analysed as described in Table 1. 100% SR-ATPase activity was 12 IU mg⁻¹. Note that the sonication procedure has little effect on the level of endogenous lipid in the isolated complexes and about 5–15% of the added DOL-cholesterol becomes fused with the SR-ATPase complexes.



Lipid composition of isolated complexes (mol per mol ATPase)

Fig. 4 Reactivation of ATPase by displacing cholesterol bound to protein by SR lipids. Complexes containing cholesterol described in legend to Fig. 3 were suspended at 5–10 mg ml⁻¹ protein in sucrose buffer containing 10 mg ml⁻¹ cholate and 20–40 mg ml⁻¹ SR lipids. After incubation at 0 °C for 2 min the mixture was diluted tenfold into sucrose buffer and incubated for a further 30 min at 0 °C before assay at 37 °C. Shaded area defines activity of complexes before addition of SR lipids; open area, reactivation produced by these added lipids. Factor by which the complexes have been reactivated is shown above each open area. Dashed lines, experiments in which sufficient SR lipid was added back to complete an annulus of exactly 30 SR phospholipid molecules. 100% SR-ATPase activity was 13.5 IU mg⁻¹

activities of the ATPase, succinate dehydrogenase and β -hydroxybutyrate dehydrogenase were all inhibited and the inhibition was fully reversed on removal of cholate. In this respect the lipid interactions of these proteins contrast sharply with the Folch–Lees protein from myelin which was found by London *et al.*⁸ to have a strong interaction with cholesterol monolayers.

Cholesterol may be inhibitory because it does not meet the requirement of the protein for specific chemical structures in the annulus: for example, β -hydroxybutyrate dehydrogenase specifically requires lecithin to bind the NAD⁺ coenzyme^{9,13}. On the other hand the very rigid interaction of cholesterol with the hydrophobic surface of the protein would also account for the inhibition of ATPase activity. The reversible inactivation of

complexes of the ATPase with saturated lecithins at or below the thermal phase transition demonstrate that a highly rigid interaction is sufficient to completely inhibit the ATPase⁶. The inhibition by both cholesterol and the saturated lecithins can be partially relieved by perturbing agents, such as short-chain alkyl alcohols, which fluidise the lipid environment of the protein¹⁰.

It is probable that entropy factors are responsible for the exclusion of cholesterol from the phospholipid annulus in the ATPase–lipid complexes. It has recently been shown by de Kruijff *et al.*¹¹ that mixtures of two lecithins which differ by four or more carbons in their fatty acid chains undergo separate phase transitions and that cholesterol is preferentially localised in the more fluid component which has a lower transition temperature. As the annular interaction of phospholipids with ATPase is substantially more rigid and immobilised than the lipid bilayer distant from the protein, a similar mechanism for the exclusion of cholesterol from the annulus is at least plausible.

Finally, we regard the lateral organisation of phospholipids and cholesterol around the protein as a specific example of the way in which the annulus is designed to optimise the accumulation of calcium by SR which will be reported elsewhere.

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- Scandella, C. J., Devaux, P., and McConnell, H. M., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 2056 (1972).
- Poo, M.-m., and Cone, R. A., *Nature*, **247**, 438 (1974).
- Hasselbach, W., and Makinose, M., *Biochem. biophys. Res. Commun.*, **7**, 132 (1962).
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 622 (1974).
- Warren, G. B., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C., *Membrane Proteins in Transport and Phosphorylation* (edit. by Azzone, G. F., Klingenberg, M. E., Quagliariello, E., and Siliprandi, N.), 1–12 (North Holland, Amsterdam, 1974).
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C., *Biochemistry*, **13**, 5501 (1974).
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C., *FEBS Lett.*, **41**, 122 (1974).
- London, Y., Demel, R. A., Geurts Van Kessel, W. S. M., Zahler, P., and Van Deenen, L. L. M., *Biochim. biophys. Acta*, **332**, 69 (1974).
- Fleischer, S., Bock, H. G., and Gazzotti, P., *Membrane Proteins in Transport and Phosphorylation* (edit. by Azzone, G. F., Klingenberg, M. E., Quagliariello, E., and Siliprandi, N.), 125–136 (North Holland, Amsterdam, 1974).
- Metcalfe, S. M., Warren, G. B., and Metcalfe, J. C., *Cell Surfaces and Malignancy* (edit. by Mora, P.) (Fogarty International Center, NIH, USA, in the press).
- De Kruijff, B., *et al.*, *Biochim. biophys. Acta*, **356**, 1 (1974).
- Jost, P. C., Griffiths, O. H., Capaldi, R. A., and Vanderkoi, G., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 480 (1973).
- Houslay, M. D., Warren, G. B., Birdsall, N. J. M., and Metcalfe, J. C., *FEBS Lett.*, **51**, 146 (1975).

letters to nature

Anisotropy of cosmic radiation in the Galaxy

ONE of the most interesting features of high energy cosmic radiation is its striking isotropy. A large degree of isotropy would be an obvious consequence of an extragalactic origin for cosmic rays, but there are some difficulties in interpreting it in terms of a galactic origin. Although there are theoretical arguments in favour of the latter, the existence of cosmic-ray anisotropy in the Galaxy has not yet been proved experimentally. Here we report a measurement, point M in Fig. 1, which proves the existence of an anisotropy in galactic cosmic radiation. It was detected by examining extensive air showers produced by cosmic-ray primaries of energies $\sim 6 \times 10^{13}$ eV (which are

certainly not affected by solar modulation).

The results of other anisotropy measurements are also shown in Fig. 1. Some of these^{1–2} give upper limits only; others^{3–5} do indicate some anisotropy. But the Hobart measurement⁴ is at low energies for which solar modulation effects are not negligible, and the residual solar waves obtained at Ithaca cast some doubts on the Ithaca points. As for the second Chacaltaya point³, its statistical weight is rather low and the statistical error given seems to be underestimated. The Pic du Midi experiment⁶ also shows some evidence for an anisotropy of about 0.1–0.15% in the energy interval 10^{13} – 10^{15} eV. In a later summary⁷, however, one of the authors claims to have found no significant sidereal wave at the Pic du Midi.

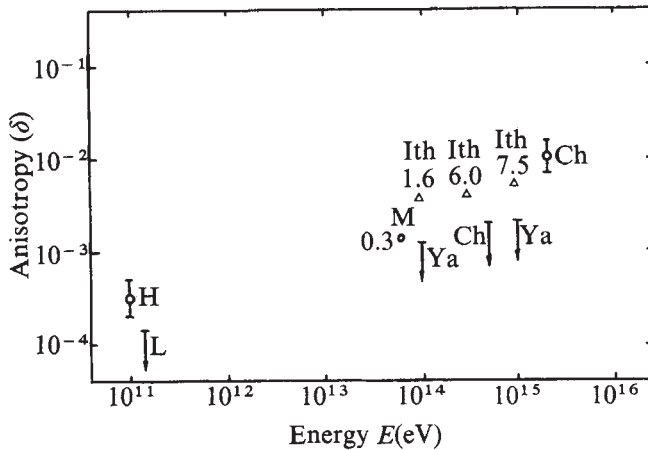


Fig. 1 A plot of experimental results concerning anisotropy, δ , of particle fluxes in the Galaxy as a function of E , the particle energy. Figures indicated near the Musala (our work) and Ithaca⁵ points are confidence levels (%). H, Hobart⁴; L, London¹; M, Musala; Ith, Ithaca⁵; Ch, Chacaltaya³; Ya, Yakutsk².

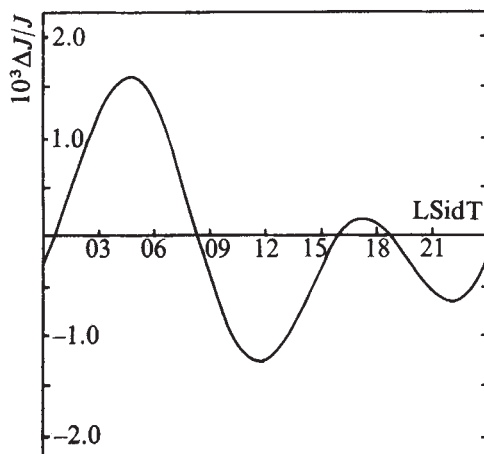
Our apparatus is on the Musala peak (42°N, 24°E) in the Rila Mountains in Bulgaria at an altitude of 2,925 m. The apparatus is of conventional design: four sets of Geiger-Müller counters, each of total sensitive area of 2.5 m², are situated at the corners of a square 8 m × 8 m and connected in four-fold coincidence. The measurement has been running since the autumn of 1968 with temporary interruptions, the largest of which lasted for six months. The counting rate is about 9,000 h⁻¹. Data obtained up to December 31, 1972 were analysed in 3-h bins. The active time of the apparatus was about 11,000 h during which time about 10⁸ showers were recorded.

The mathematical method used in the data analysis consisted of a multiple Fourier and simultaneous regression analysis, including barometric pressure and atmospheric temperature. The first three harmonics of solar, sidereal and anti-sidereal daily variations were considered; the flux of the extensive air showers observed was assumed to be of the form

$$J(t) = A + B\Delta p + C\Delta T + \sum_{j=1}^3 \sum_{k=1}^3 \{a_{jk} \cos(k\omega_j t) + b_{jk} \sin(k\omega_j t)\}$$

where Δp and ΔT are the deviations of the barometric pressure

Fig. 2 Sum of the first two harmonics in local sidereal time of the pressure and temperature corrected data obtained in the Musala experiment. $\sim 10^8$ showers; $E_0 \approx 6 \times 10^{13}$ eV.



and atmospheric temperature, respectively, from their mean values. The temperature was measured at the $p_0 - 120$ mbar level where p_0 is the actual (variable) value of the barometric pressure taken at the level of observation. The ω_j ($j = 1, 2, 3$) represent the solar, sidereal and anti-sidereal daily frequencies, respectively. Values of the 21 parameters A , B , C , a_{jk} and b_{jk} , together with their errors and correlation coefficients, were estimated simultaneously by the method of maximum probability.

The relative amplitudes of the solar and anti-sidereal waves remained below noise level (which was roughly 3×10^{-4}). The first two harmonics of the sidereal wave were significant, the confidence level for their superposition exceeding 99.7%. The first harmonic was 7.77×10^{-4} with maximum at 3 h 25 min local sidereal time (LSidT) and the second harmonic had an amplitude of 8.71×10^{-4} with maxima at 5 h 00 min and 17 h 00 min LSidT. The third harmonic was below noise level.

The sum of the first and second harmonics is shown in Fig. 2 and the galactic directions scanned by the apparatus in Fig. 3. There is a maximum between 2400 and 0800 LSidT. In this time interval the detector looks at directions with small galactic declinations; the directions of large fluxes seem to be centred around the galactic anti-centre rather than the inward direction of the galactic arm. There is a minimum between 0800 and

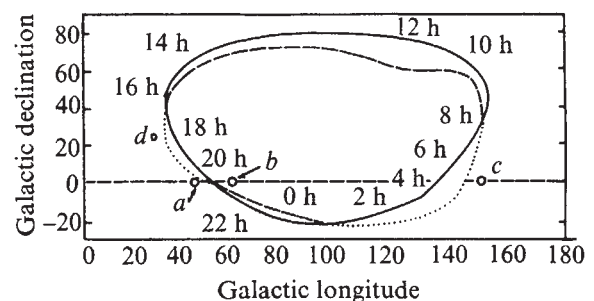


Fig. 3 Galactic directions scanned by the Musala apparatus. Dotted and dashed lines indicate regions of maximal and minimal intensities, respectively. a, Direction of spiral in; b, direction of galactic rotation; c, direction of galactic anticentre; d, direction of apex motion.

1600 LSidT. In this interval directions close to the galactic North Pole are scanned by the apparatus, and the time interval between 1600 and 2400 LSidT is rather flat.

We note that an isotropic angular distribution of cosmic rays in the Galaxy would result in small sinusoidal solar and sidereal variations because of the orbital motion of the Earth and the apex motion of the Solar System, respectively. Both of these variations are below noise level, at $4.5 \times 10^{-4} \cos \lambda$ and $3 \times 10^{-4} \cos \lambda$, respectively, where λ is the geographical latitude of the observation. The anisotropy indicates that at least some cosmic-ray primaries with energies of about 6×10^{13} eV are of galactic origin.

The absence of a surplus intensity from the direction of the galactic spiral arm indicates that there is no streaming of cosmic rays along the spiral arm at velocities > 40 km s⁻¹, which would give rise to a peak in the intensity of the order of 3×10^{-4} .

The intensity maximum observed from the direction of the galactic anti-centre may be understood in terms of an increased density of cosmic rays within the galactic arm. If the Solar System is near the inner edge of the Orion spur then cosmic-ray diffusion produces an anisotropy similar to that observed. On the other hand, we can not exclude the possibility that the anisotropy detected is induced by streaming of cosmic rays in

directions out from the Galaxy and perpendicular to the galactic plane (the Sun being to the north from the galactic plane).

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- ¹ Speller, R., Thambyahpillai T., and Elliot, H., *Nature*, **235**, 25–29 (1972).
² Efimov, N. N., Krasilnikov, D. D., Nikolskii, S. I., and Shamsutdinova, F. K., *Can J. Phys.*, **46**, S84–86 (1968).
³ Escobar, I., Nerurkar, N., and Weil, R., *Planet. Space Sci.*, **1**, 155–60 (1959).
⁴ Jacklyn, R. M., *ANARE Sci. Rep.*, No. 114 (1970).
⁵ Delvaile, J., Kendzioriski, F., and Greisen, K., *J. phys. Soc. Jap.*, **17**, Suppl. III, 76–83 (1962).
⁶ Daudin, J., et al., *Nuovo Cim.*, **3**, 1017–32 (1956).
⁷ Cachon, A., *Proc. VIth Interamerican Seminar on Cosmic Rays*, **2**, 39–42 (1962)

Structure of irregular galactic magnetic fields

ALTHOUGH there are various methods of investigating experimentally the integral properties of galactic magnetic fields (see, for example, ref. 1); little is known experimentally of the nature of the random component of these fields, at least as far as structures with characteristic lengths $\lesssim 1$ pc are concerned. Here I shall describe a method which, supposing the diffusion approximation of cosmic-ray propagation to be valid, is suitable for determining the structural composition of random galactic fields on the basis of cosmic-ray measurements, down to structures with characteristic lengths ~ 0.001 – 1 pc.

Magnetic fields, the direction and magnitude of which vary randomly in space, can be characterised by their power spectra, that is, by the function

$$M(k) = \int_{-\infty}^{\infty} C(r) \exp(-i2\pi rk) dr$$

where $C(r)$ is the coherence function of the random field. I shall show that the form of $M(k)$ can be determined on the basis of the functions $a(E)$, the anisotropy of galactic cosmic rays as a function of energy, and $L(E)$, the total path length of galactic cosmic rays as a function of energy. I shall attempt to apply the procedure on the basis of our present knowledge of the functions $a(E)$ and $L(E)$.

The energy range of the cosmic-ray particles considered is 10^{12} eV $\lesssim E \lesssim 10^{15}$ eV. The lower limit ensures that the energy density of the particle radiation will be low enough to leave the magnetic field traversed unaffected and excludes the possibility that the observed cosmic-ray anisotropy is produced by interplanetary magnetic fields. The upper energy limit excludes regions in which the Larmor radii of cosmic-ray particles may approach the sizes of regions of magnetic irregularities, thus making diffusion processes less effective.

I shall proceed by deriving the energy dependence of λ_p , the diffusion mean free path of cosmic-ray particles in the Galaxy. For simplicity, I assume that the galactic cosmic radiation consists exclusively of protons. There is no intrinsic difficulty in repeating the process for the actual chemical composition of the galactic radiation, except that the chemical composition is very poorly known in the energy interval 10^{12} eV $\lesssim E \lesssim 10^{15}$ eV.

For the simple diffusion process, the fundamental relations between a , L , λ_p and D (the latter being the linear size of the volume to which particles are confined) are

$$D = aL \quad (1)$$

and

$$\lambda_p = a^2 L \quad (2)$$

In the case of the so-called “compound” diffusion process², equation (2) has to be replaced by

$$\lambda_m^2 \lambda_p = a^4 L^3 \quad (3)$$

where λ_m is the characteristic length (mean free path) of the ‘random walk’ of magnetic field lines. In equations (1), (2) and (3) constant factors of the order of unity are neglected.

The functions $a(E)$ and $L(E)$ can, in principle, be determined experimentally. In the preceding paper³ a recently measured value of $a(E)$ at $E \approx 6 \times 10^{13}$ eV was reported. Similar measurements, if carried out at various energies, may yield the form of $a(E)$, whereas $L(E)$ can be evaluated on the basis of measurements of the chemical composition of galactic cosmic rays^{4–6}.

Although serious objections to the simple diffusion model have been raised² and the results of Gombosi *et al.* also seem to favour the compound model rather than the simple one⁷, I shall investigate the consequences of both models, since they yield the same form for $M(k)$ provided that D does not depend on E .

As to the energy dependence of λ_p , the simple diffusion model obviously yields

$$\lambda_p(E) \sim a^2(E)L(E) \quad (4)$$

In the case of the compound model an assumption has to be made as to the form of $\lambda_m(E)$. I shall suppose that λ_m is independent of E . This seems reasonable on the basis of the assumptions made in the compound diffusion model, in particular that particles are spiralling along field lines while the latter are undergoing random motion in space.

From equation (3) I then have

$$\lambda_p(E) \sim a^4(E)L^3(E) \quad (5)$$

I now apply the formula of Jokipii⁸ according to which for high energy protons (particle rigidity and particle energy may be interchanged)

$$\lambda_p = E^2/\pi M(k) \quad (6)$$

and $M(k)$ has to be taken at

$$k = 1/r_g(E) \sim B/E \quad (7)$$

where $r_g(E)$ is the gyroradius of a particle with energy E in the magnetic field B in question (resonance scattering).

Equations (4), (6) and (7) yield

$$\text{Simple diffusion: } M(B/E) \sim E^2/a^2(E)L(E) \quad (8)$$

whereas equations (5), (6) and (7) give

$$\text{Compound diffusion: } M(B/E) \sim E^2/a^4(E)L^3(E) \quad (9)$$

Equations (8) or (9) yield the form of $M(k)$. If $a(E)$ and $L(E)$ are power functions of E , that is

$$a(E) \sim E^\eta \text{ and } L(E) \sim E^{-\beta} \quad (10)$$

then $M(k)$ too will be a power function, since according to equations (8) and (10)

$$\text{Simple diffusion: } M(k) \sim k^{-(2+\beta-2\eta)} \quad (11)$$

and according to equations (9) and (10)

$$\text{Compound diffusion: } M(k) \sim k^{-(2+\beta-4\eta)} \quad (12)$$

Assuming the average field strength in the Galaxy to be $\sim 10^{-6}$ gauss, protons of energies 10^{12} – 10^{15} eV will have gyroradii ~ 0.001 – 1 pc. Equations (8), (9), (11) and (12) may thus be applied in the region where

$$0.001 \text{ pc} < k^{-1} < 1 \text{ pc}$$

It is usual to assume $M(k)$ to be of the form $M(k) \sim k^{-\alpha}$ so equations (11) and (12) give the following expression for α in terms of η and β :

$$\text{Simple diffusion: } \alpha = 2 + \beta - 2\eta \quad (13)$$

$$\text{Compound diffusion: } \alpha = 2 + 3\beta - 4\eta \quad (14)$$

Present experimental results do not enable definite conclusions to be drawn about the values of η and β , that is, the energy dependence of anisotropy and total path length of galactic cosmic rays, respectively. The following crude estimates can, however, be obtained.

On the basis of Fig. 1 of Gombosi *et al.*³ one may tentatively assume η values like

$$0.3 \lesssim \eta \lesssim 0.5 \quad (15)$$

This estimation is supported mainly by the result of the measurement reported there. Smaller values of η are improbable, for otherwise the London measurement⁹ carried out at rigidities around 10^{11} V would have revealed a definite anisotropy instead of an upper limit only, and larger values of η are improbable on similar arguments concerning rigidities of $\sim 10^{15}$ V (compare Fig. 1 of Gombosi *et al.*³).

On the basis of Fig. 3 of ref. 6 one may tentatively assume

$$0.1 \lesssim \beta \lesssim 0.3 \quad (16)$$

in the energy range $1 \lesssim E \lesssim 50$ GeV. Nothing is yet known of the value β in the energy range 10^{12} eV $\lesssim E \lesssim 10^{15}$ eV. Assuming, somewhat arbitrarily, that equation (16) remains valid also in this energy range, and putting

$$\eta = 0.4 \pm 0.1 \text{ and } \beta = 0.2 \pm 0.1 \quad (17)$$

one obtains on the basis of equation (13)

$$M(k) \sim k^{-(1.4 \pm 0.2)}$$

and of equation (14)

$$M(k) \sim k^{-(1.0 \pm 0.5)}$$

From equations (1) and (10), one has

$$D(E) \sim E^{-\eta-\beta}$$

and, with respect to equation (17)

$$D(E) \sim E^{0.2 \pm 0.15}$$

that is, D , the linear size of the confinement volume depends but weakly on E . Complete independence would prevail if $\eta = \beta$. Present estimates (equation (17)) of η and β do not exclude this possibility; the uncertainties are, however, very large.

I shall, however, assume that D does not depend on E . Equations (13) and (14) show that in this case both equations lead to

$$\alpha = 2 - \eta = 2 - \beta = 1.7 \pm 0.1$$

that is, both the simple and compound diffusion models yield the same form of $M(k)$. Furthermore, it is interesting to note the agreement of this value with that of $\alpha = 5/3$ imposed by Skilling *et al.*¹⁰ on theoretical grounds.

The applicability of the method described above for the determination of $M(k)$ depends essentially on the validity of the diffusion approximation in describing the propagation of cosmic rays of 10^{12} – 10^{15} eV in the Galaxy. Various types of diffusion models may lead to various forms of equations (1) and (2). Two such possibilities ("simple" and "compound" diffusion) have been dealt with in this paper. The validity of equation (6) depends on the role of resonance scattering which, however, may be less important than previously thought¹¹. But once the correct forms of equations (1), (2) and (6) are established, the method of determining $M(k)$ can be applied. Calculations carried out on the basis of the very crude experimental data available at present yield sensible results for the form of $M(k)$. Furthermore, there is some indication that the calculated form of $M(k)$ is not too sensitive to details of the diffusion process, that is, the functions $a(E)$ and $L(E)$ may contain (almost) all the information necessary to determine $M(k)$.

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¹ Verschuur, G. L., *Interstellar Gas Dynamics* (edit. by Habing, H. J.), 150–167 (Reidel Dordrecht, 1970).

² Lingenfelter, R. E., Ramaty, R., and Fisk, L. A., *Astrophys. Lett.*, **8**, 93–97 (1971).

³ Gombosi, T., *et al.*, *Nature*, **255**, 687–689 (1975).

⁴ Webber, W. R., Lezniak, J. A., Kish, J. C., and Damle, S. V., *Nature*, **241**, 96–98 (1973).

⁵ Audouze, J. M., and Cesarsky, C. J., *Nature*, **241**, 98–100 (1973).

⁶ Ramaty, R., Balasubrahmanyam, V. K., and Ormes, J. F., *Science*, **180**, 731–733 (1973).

⁷ Gombosi, T., *et al.*, *Collected Papers of the 14th International Conference on Cosmic Rays, München* (in the press).

⁸ Jokipii, J. R., *Astrophys. J.*, **146**, 480–487 (1966).

⁹ Speller, R., Thambyahpillai, T., and Elliot, H., *Nature*, **235**, 25–29 (1972).

¹⁰ Skilling, J., McIvor, I., and Holmes, J. A., *Mon. Not. R. astr. Soc.*, **167**, 87–91 (1974).

¹¹ Webb, S., and Quenby, J. J., *Solar Phys.*, **37**, 235–249 (1974).

Hypothesis for the Type I supernova light curve

IN a Type I supernova event^{1–3} the observed luminosity rises very rapidly to a maximum absolute visual magnitude of about -19.0 ± 0.3 . After about 20 d the light curve (Fig. 1) can be characterised by two exponential decays with half lives τ_1 and τ_2 where τ_1 is of the order of several days and τ_2 varies approximately from 40 to 60 d. The integrated observed luminosity is similar for all Type I supernova and is about 3.6×10^{49} erg. A feeble but persistent departure from a simple double exponential decay is also apparent in Fig. 1 and disappears on a time scale τ_3 of ~ 80 d. We suggest that a model involving ⁵⁶Ni white dwarf can explain these changes.

Because of its exponential nature it has been suggested that the light curve is associated with the decay in the remnant of explosively produced radioactive species^{4,5} such as ²⁵⁴Cf and ⁵⁶Ni. These hypotheses have fallen into disfavour because the exponential decay times vary substantially from event to

event and also because the interstellar medium would become overabundant in heavy elements produced by this process. But our attention has been refocused on such a possibility by the observations which Van Hise⁶ made of the supernova in IC4182. He measured $\tau_1=4.8$ d and $\tau_2=56$ d for this event and pointed out that these numbers bear an interesting relationship to the decay sequence of ^{56}Ni . Terrestrially, ^{56}Ni beta decays to ^{56}Co entirely by electron capture with a half life of $\tau_{\text{Ni}}=6.1$ d. In turn the ^{56}Co beta decays to ^{56}Fe with a half life $\tau_{\text{Co}}=77$ d, 80% by electron capture and 20% by positron emission. The ^{56}Ni decay releases an average total of 1.72 MeV in the form of gamma rays compared to 3.45 MeV for the ^{56}Co decay. Van Hise observed that for the event in IC4182 $\tau_1/\tau_{\text{Ni}} \approx \tau_2/\tau_{\text{Co}} \approx 0.75$. In other words the light curve looks very much like the ^{56}Ni - ^{56}Fe decay sequence but at an enhanced rate. This observation is even more suggestive in the light of explosive nucleosynthesis calculations^{7,8} which indicate that the most abundant radioactive element produced by Type I supernovae may be ^{56}Ni with $\sim 0.1 M_\odot$ per event being a reasonable mass estimate.

We propose that each Type I supernova event leads to the formation of a fully convective white dwarf star containing $\sim 0.2 M_\odot$ of ^{56}Ni with a $\sim 1 M_\odot$ remnant expanding away from the star with a velocity⁹ $\sim 20,000$ km s⁻¹ which contains a lesser amount of ^{56}Ni . This hypothesis leads in a simple way to an understanding of the data. The essential idea is that the light curve is powered by the ^{56}Ni decay sequence at an enhanced rate because of the effect of the great matter density in a white dwarf. The electron capture process is proportional to the electron density ρ_e at the nucleus. At ordinary densities the decay of a ^{56}Co or ^{56}Ni nucleus is dominated by the capture of an inner K-shell electron and is relatively insensitive to the state of ionisation or chemical bonding. Using the Hartree-Fock wave functions tabulated by Herman and Skillman¹⁰ we obtain $\rho_e=8.6 \times 10^{28}$ electrons cm⁻³ for ^{56}Co and $\rho_e=9.7 \times 10^{28}$ electrons cm⁻³ for ^{56}Ni in ordinary matter. In a white dwarf ρ_e will be increased above the terrestrial values if the average bulk density $\bar{\rho}_{\text{av}}$ is comparable to 10^{29} electrons cm⁻³. The picture here is one of bare nuclei moving through a Fermi sea of degenerate electrons experiencing the average bulk electron density $\bar{\rho}_{\text{av}}$. This picture is not perfect since the positive nuclei will tend to attract electrons, the electron density at the nuclei will be increased by some factor above $\bar{\rho}_{\text{av}}$. At high electron densities, ρ_e at a cobalt nucleus may be estimated using non-relativistic self-consistent field linear response theory¹¹ yielding $\rho_e=\bar{\rho}_{\text{av}}(1+0.60 \times 10^{10} \times \bar{\rho}_{\text{av}}^{-1/3})$ electrons cm⁻³. This formula fails where it yields results less than the terrestrial density because K-shell binding occurs. The true value of ρ_e is estimated by smoothly joining these results (see Fig. 2). This gives $\rho_e=1.9 \times 10^{29}$ electrons cm⁻³ for a matter density $\rho_m \approx 2.4 \times 10^5$ g cm⁻³ and corresponds to a half life of 40 d. This important result may be somewhat modified after more detailed calculations.

Fig. 1 A schematic Type I supernova light curve.

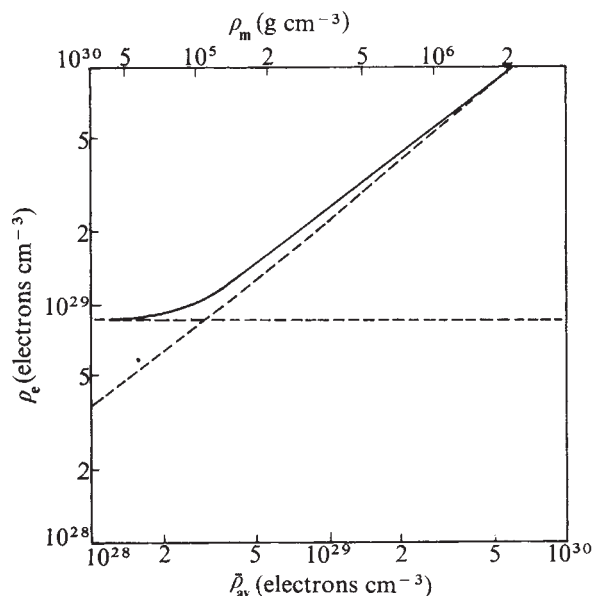
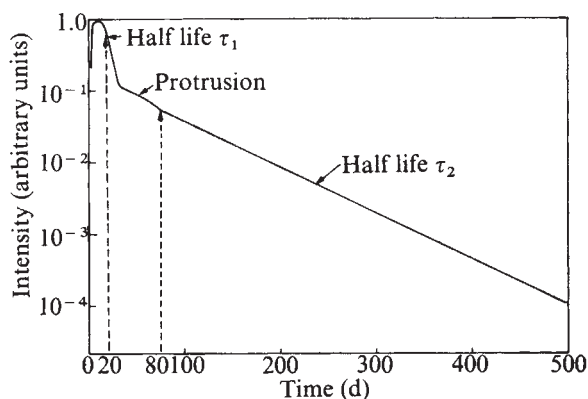


Fig. 2 A plot of the average bulk electron density in a white dwarf $\bar{\rho}_{\text{av}}$ as function of the average electron density at a Co nucleus. The linear self-consistent field (LSCF) approximation is used to calculate ρ_e at large $\bar{\rho}_{\text{av}}$ (diagonal dashed line). A simple cubic lattice of Co nuclei is assumed. The solid curve is an extrapolation used to make estimates in this paper; the horizontal line marks terrestrial density.

When $\bar{\rho}_{\text{av}}$ drops below $\sim 10^{28}$ electrons cm⁻³ it is expected that K shell electrons will become bound to the nuclei and dominate the decay. So τ_1 and τ_2 are not expected to become longer than τ_{Ni} and τ_{Co} . Moreover, for an electron density giving $\tau_2=56$ d, the ratios τ_1/τ_{Ni} and τ_2/τ_{Co} should be the same to within 3% when one considers the facts that 80% of ordinary ^{56}Co is being converted by electron capture but that it has a 12% lower electron density than ^{56}Ni .

Since the matter density is expected to vary significantly over the volume of the star one might expect the observed decay rate to be an integral sum of a series of exponentials which itself may or may not seem exponential. But if the star is fully convective on the time scale of $\lesssim 1$ d then the decay would indeed seem exponential at a rate corresponding to the average electron density at the cobalt nucleus.

More heat per unit mass is generated in the centre of the star than elsewhere, because in the centre the mass density, and thus the decay rate, is greatest. Excess heat will be transported out either by conduction or convection. The convective velocity in the core of a degenerate star has been estimated to be about $10^3 (L/L_\odot)^{1/3}$ cm s⁻¹ (refs 12 and 13). Since the luminosity L varies from about 10^{10} to $10^6 L_\odot$, a convective mixing time for the present case is about 30 min initially, and several hours after 500 d, thermal conduction times are surely longer, so that violent convection should occur.

No Type I events have been observed with a $\tau_2 \lesssim 40$ d, suggesting that there is an upper mass limit on the ^{56}Ni dwarf formed by the supernova. A τ_2 of 40 d corresponds to an average $\rho_m \approx 2.4 \times 10^5$ g cm⁻³. For a cold white dwarf¹⁴ we find $M \lesssim 0.4 M_\odot$. This result should be insensitive to chemical composition. But effects not included here (such as rotation and high temperatures) may modify this result considerably. The τ_2 values do not set a lower limit on M , but the integrated observed luminosity does. If the energy released in the radioactive decay is converted to visible light with 100% efficiency then the average dwarf must contain $\sim 0.2 M_\odot$ of ^{56}Ni .

Because of the rapid convectivity the energy escapes from the star fast enough to allow the light curve to follow the exponentials. Energy emitted from the surface would be a combination

of black body photons and energetic particles. If the light curve is to follow accurately an exponential a net mass loss $\lesssim 10\%$ over 500 d is required. Since the peak luminosity of a Type I supernova is about 1.2×10^{43} erg s⁻¹ the characteristic photon energy is in the soft X-ray region. The remnant should then remain opaque because of ionisation, Compton scattering, and inelastic collisions. Thus we believe that the energy is rapidly deposited in the remnant in the form of electron kinetic energy and converted to visible light by gas collision processes. The diffusion times for visible photons out of the remnant are short enough not to distort the exponential decay.

One convenient explanation of the feeble protrusion in the light curve which disappears on a time scale ~ 80 d is provided by a small amount of ⁵⁶Ni and ⁵⁶Co decaying in the remnant. The originally compact remnant is initially opaque to the emitted gamma rays and this energy will also be converted to electron kinetic energy by the Compton process. But as the remnant continues to expand it will eventually become transparent to the gamma rays from the decay. The total absorption coefficient of a 0.5 MeV gamma ray in typical matter is ~ 3 g cm⁻² and the column density of the remnant falls to ~ 1 g cm⁻² after 80 d.

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- ¹ Zwicky, F., in *Stellar Structure*, 367 (edit. by Aller, L. H., and McLaughlin, D. B.) (University of Chicago Press, 1968).
- ² Minkowski, R., in *Supernova and Their Remnants*, 23 (edit. by Branchio, P. J., and Cameron, A. G. W., Gordon and Breach, New York, 1969).
- ³ Shklovsky, I. S., *Supernova* (Wiley-Interscience, London and New York, 1968).
- ⁴ Baade, W., et al., *Publ. astr. Soc. Pacific*, **68**, 296 (1956).
- ⁵ Colgate, S. A., and McKee, C., *Astrophys. J.*, **157**, 623 (1969).
- ⁶ Van Hise, J. R., *Astrophys. J.*, **192**, 657 (1974).
- ⁷ Bodansky, D., Clayton, D. D., and Fowler, W. A., *Astrophys. J. Suppl.*, **16**, 299 (1968).
- ⁸ Clayton, D. D., in *Explosive Nucleosynthesis*, 264 (edit. by Schramm, D. N., and Arnett, W. D., University of Texas Press, Austin, 1973).
- ⁹ Colgate, S. A., and White, R. H., *Astrophys. J.*, **143**, 626 (1966).
- ¹⁰ Herman, F., and Skillman, S., *Atomic Structure Calculations* (Prentice Hall, Englewood Cliffs, 1963).
- ¹¹ Kittel, C., *Quantum Theory of Solids*, (Wiley, New York, Chapter 6, 1963).
- ¹² Paczynski, B., *Astr. Lett.*, **11**, 53 (1972).
- ¹³ Ruderman, M. A., and Sutherland, P. G., *Nature*, **246**, 93 (1973).
- ¹⁴ Van Horn, H. M., *Astrophys. J.*, **151**, 227 (1968).

Shock-induced remanent magnetisation in late Precambrian rocks from Lake Superior

A POSSIBLE shock-induced remanent magnetisation, the direction of which yields a typical 'Grenville' pole position has been discovered from late Precambrian Keweenawan igneous rocks (about 1,100 Myr old) on the Slate Islands, situated in northern Lake Superior about 300 miles north-west of the Grenville Front.

The Slate Islands form a cluster of small islets primarily composed of strongly foliated Archaean extrusive and pyroclastic rocks. Along the western shore of Patterson Island, the largest of the group, there are about 450 feet of Keweenawan mafic volcanics, comprising about 20 flows (my unpublished data) that are lithologically similar to other Keweenawan extrusives which border and underlie much of Lake Superior^{1,2}. On the Slate Islands the volcanics dip south-west about 30–40°, although toward the base of the sequence dips as great as 50–70° occur. Diabase dikes, trending east to north-east, occur in profusion throughout

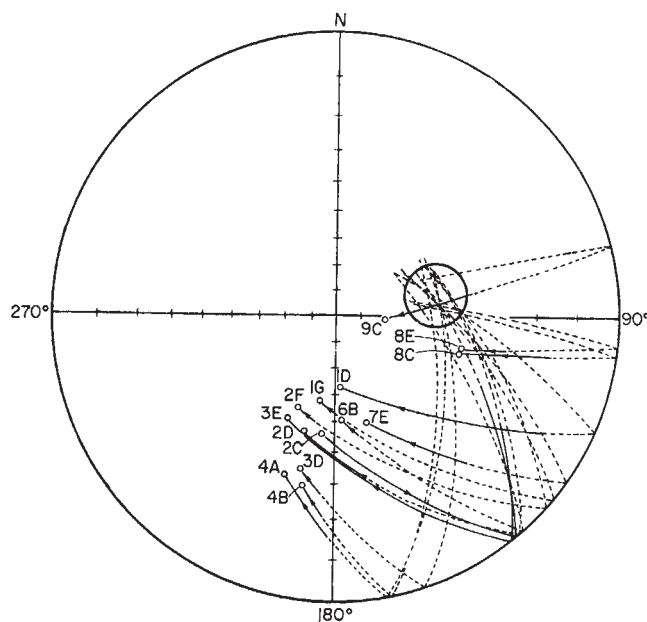


Fig. 1 Wulff stereonet showing remagnetisation circles of samples which yield stable end-points. Some samples have been omitted to avoid congestion. Arrows indicate sense of directional swings on stepwise a.c. cleaning. Data uncorrected for geological dip. ○, Stable end-point (negative inclination); —, measured curve; ---, extrapolated curve. Large circle indicates approximate area of convergence.

the islands and are presumably feeders for Keweenawan flows.

Two unusual features of Slate Islands geology are a red breccia which cuts all rocks including Keweenawan flows, and conical fractures, known as shatter cones³ which are generally thought³⁻⁵ to be shock-induced. The breccia occurs throughout the islands as veins ranging in thickness from less than an inch to several feet, and carries angular to subrounded fragments of host rocks set in a matrix of comminuted rock⁶. Shatter cones are found in both Keweenawan and older rocks from the western half of the Slate Islands but have yet to be recognised in the east where only highly cleaved Archaean rocks occur (R. P. Sage, personal communication). The cones are particularly conspicuous in the Keweenawan flows where they point eastward and have axes inclined about 10° to the east (ref. 6 and R. P. Sage, personal communication).

More than 60 samples were collected at 12 sites from Keweenawan flows and dikes, and oriented using a Sun compass (accuracy $\pm 2^\circ$). Three samples were also taken from a red breccia zone. Samples were cored in the laboratory and specimen remanences measured using a spinner magnetometer (sensitivity 0.02×10^{-6} e.m.u. cm⁻³). At least one specimen per sample was a.c. demagnetised in steps of 50 or 100 oersted up to maximum peak fields of 1,000 oersted.

Two magnetisation components, A and B, were recognised in all but three specimens on stepwise cleaning, as remanent directions smoothly followed great circle paths on the stereonet (Fig. 1). In general, directions progressively moved from smaller to greater declinations and from positive to negative inclinations. About half the specimens attained stable end-points corresponding to remanence A (Table 1). If the circles are extrapolated to lower inclinations, their point of convergence gives the direction of the second magnetisation component B.

The direction of the B remanence was found through use of variational methods⁷ to fit a least squares plane,

Table 1 Site palaeomagnetic data for remanence A

Site	N	D ₁	I ₁	D ₂	I ₂	k	α_{95}	H
1	5	192.1	-59.2	135.9	-61.6	287	4.5	6-10
2	5	196.8	-48.8	145.1	-63.1	168	5.9	6-9
3	5	200.9	-38.4	173.6	-57.3	46	11.4	6-10
4	2	194.9	-30.0	174.5	-47.6	195	12.1	7-10
5	3	208.1	-44.0	152.6	-70.8	256	7.7	7-10
6	1	173.0	-54.0	120.3	-55.1	—	—	4-6
7	1	162.0	-50.0	124.5	-42.2	—	—	6-9
8	2	104.9	-41.5	104.9	-41.5	4,045	3.9	5-7
9	2	99.9	-59.0	99.9	-59.0	26	50.9	4-6
Mean Site	9			135.0	-58.2	20	11.8	

Pole position for A remanence: 183°W; 56.6°N.

N=Number of samples in which stable end points have been found. A stable end point is defined if the magnetisation direction remains constant within $\pm 1^\circ$ over at least 3 successive a.c. demagnetisation field increments spanning at least a range of 200 oersted. H is the average range of peak demagnetising field (in oersted $\times 100$) over which stability is observed. D₁, I₁ and D₂, I₂ are respectively declination and inclination before and after structural correction, in degrees. k and α_{95} are respectively the Fisher precision parameter and the half angle of the 95% confidence cone.

firstly for all remanence vectors defining each sample remagnetisation circle, and then, after averaging to the site level, for all normals to site remagnetisation circles (Fig. 2). The normal to the least squares plane in Fig. 2 yields a direction D=85.5°, I=47.5° for remanence B.

All data used to define remagnetisation circles (Table 2) exclude natural remanent magnetisation (NRM) directions or those obtained after a.c. cleaning to 50 or 100 oersted. Below 100 oersted, viscous magnetisation components are often present. Once they are removed, remanence directions seldom depart more than 1°–2° from remagnetisation planes during a.c. cleaning. Larger deviations were only observed in some dike specimens, after the remanence had fallen below about 10% of the initial intensity.

When the data in Fig. 1 are referenced to the palaeohorizontal, remanent vectors, defined by stable end points, yield a mean direction (Table 1) which lies within about 10° of that usually found⁸⁻¹⁰ for reversely magnetised Keweenaw igneous rocks (Fig. 3), and which is attributed to a TRM formed during initial magma cooling. Remanence A is thus considered to be primary (Keweenaw). Remanence B must therefore be a secondary component, as suggested also by the observation that in diabase dikes, the B component is dominant in coarse-grained interiors but almost absent in chilled margins where the smaller magnetic grains are likely to have higher coercivities and blocking temperatures and thus be more resistant to remagnetisation. The B remanence, moreover, seems to have postdated differential tilting in the Keweenaw volcanics, because maximum convergence of remag-

netisation circles is obtained before any structural corrections are made (Figs 1, 2).

The Slate Islands are unique in that no other Keweenaw extrusive sequence around Lake Superior possesses either shatter cones or a hard prominent secondary remanence. It seems reasonable that the features are related and were acquired during a shock event of presently unknown nature. The remarkable convergence of the remagnetisation circles, considering that maximum orientation errors are possibly 5° after laboratory coring, suggests that the B remanence, unlike the A, was acquired over a time interval short compared with time scales of secular variation. Furthermore, the direction of shatter cones is uniform throughout the Keweenaw sequence and does not reflect differences in structural attitude of flows (R. P. Sage, personal communication). The shatter coning and secondary remanence thus postdate differential tilting, an observation which enhances the idea of a common cause of both.

The red breccia is probably also a product of the shock event, but this conclusion cannot be verified with the scanty palaeomagnetic data available. Of the three breccia samples that were a.c. cleaned, one gave a stable B remanence direction, but the other two, although exhibiting dominant B components, were unstable.

The shock event is suspected to be a meteorite impact because shatter cones are commonly observed in association with rocks whose origin cannot be readily ascribed to normal geological processes³, and may be formed by shock pressures greater than 20 kbar, values considerably higher

Table 2 Summary of palaeomagnetic data showing remagnetisation circles

Site	N	n	D° ^M	I°	k	α_{95}
1	9	11	30.4°	-29.4°	94	5.3°
2	5	8	48.0	-37.7	400	4.6
3	5	6	62.7	-41.2	31	13.8*
4	3	7	79.5	-37.4	2,302	2.4
5	4	7	46.2	-47.7	92	9.6
6	5	4	44.5	-32.2	204	4.7
7	5	5	28.9	-29.4	469	3.5
8	5	9	7.4	-11.7	272	4.6
9	5	6	-3.4	-01.9	102	7.6
10	3	7	17.8	-18.8	293	7.1
11	5	5	-11.4	+09.3	100	6.7
12	5	10	27.2	-21.7	24	15.8*

N, Number of independently oriented samples which show clear remagnetisation circles.

n, Average number of data points per sample remagnetisation circle.

M, Mean direction of normals to sample remagnetisation circles (normal to site remagnetisation circle).

k, α_{95} , Fisher statistical parameters for the direction M.

*The large confidence limits arise because at these sites, normals to sample remagnetisation circles are themselves distributed along a plane very close to that given by the dotted line in Fig. 2 (that is the convergence of sample remagnetisation circles to the remanence B direction is even apparent at the within site level).

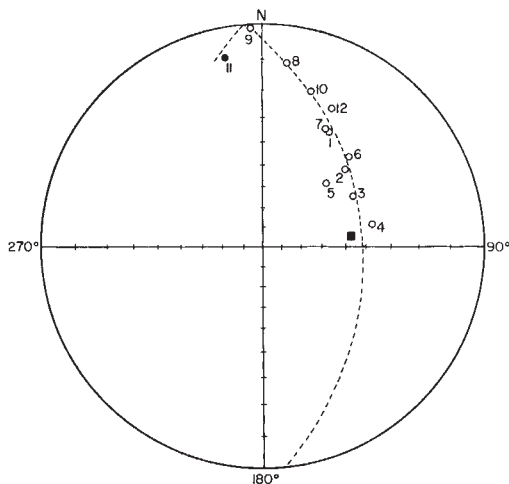
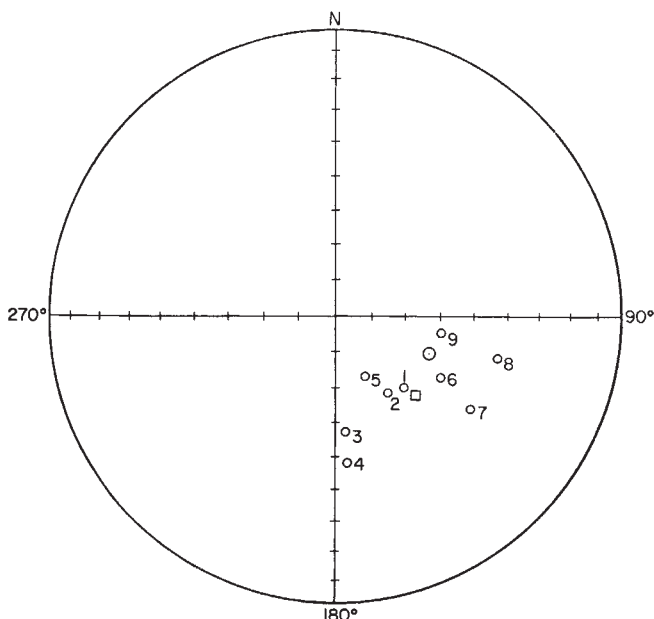


Fig. 2 Wulff stereonet showing the position and normal of the least squares plane fitted to normals of site remagnetisation circles given in Table 2. Solid (open) symbols are plotted on the lower (upper) hemisphere of the stereonet. Data uncorrected for geological dip. ○, ●, Normals to site remagnetisation circles; ---, least squares plane through normals; ■, normal to least squares plane (direction of remanence B) with $D=85.5^\circ$, $I=47.5^\circ$.

than the 3–5 kbar maximum estimated for volcanic explosions^{3,4}. The attitude of shatter cones along the western side of Patterson Island indicates that the shock centre lay somewhere to the east. The complex geology of the Slate Islands and the presence of a breccia not found on the mainland (R. P. Sage, personal communication), suggest that the shock event occurred locally. Bathymetric charts show a lakefloor depression which almost completely surrounds the islands, inviting speculation that the islands themselves are formed by the central uplift of a meteorite crater, and thus represent the site of impact.

Fig. 3 Equal area projection of site remanence A directions. Data corrected for geological dip. □, Site mean magnetisation direction for Slate Islands volcanics ($D=135^\circ$, $I=58.2^\circ$); ○, Average Keweenawan reversed magnetisation direction.



Several palaeomagnetic studies on breccias and remelted rocks associated with impact sites show^{11–14} very stable remanences with low directional dispersion. I am not aware, however, of any report describing a magnetically hard shock remanence in rocks which predate a shock event and which have retained their primary texture. Acquisition of a hard stable remanence in such rocks may either result from mobilisation of domain walls through application of high pressure, or from cooling following a sudden rise in temperature caused by fracturing and adiabatic compression during the passage of the shock wave. The B remanence direction seems unrelated to that of the shatter cone axes and hence to the shock front. It is thus likely to have formed parallel to the ambient palaeomagnetic field.

Palaeomagnetic studies on rocks from the Grenville Province^{15–17} reveal stable magnetisations which are believed to be the result of metamorphism and subsequent cooling through magnetic oxide blocking temperatures. Irving *et al.*¹⁵ have summarised all recent data and conclude that the formation of stable Grenville magnetisations occurred about 1,150–1,000 Myr ago, a time interval represented by rocks in other parts of North America (such as the Keweenawan). The Grenville polar wandering path,

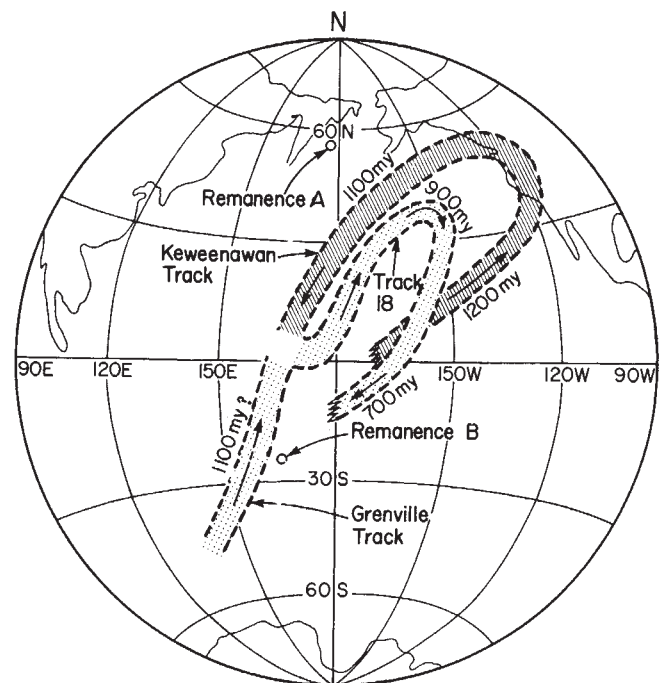


Fig. 4 Map showing the position of the remanence A and B poles relative to the polar wander tracks described¹⁵ for the time interval 1,200–700 Myr ago. The A pole does not lie directly on the Keweenawan polar path, possibly because the very thin flow sequence on the Slate Islands represents a period of time too short for complete averaging out of secular variation.

however, is very different from that of supposedly contemporaneous Keweenawan units (Fig. 4) and it has been proposed¹⁵ that Grenville magnetisations were acquired when the Grenville Province was separated from the rest of North America. If remanence B records a palaeofield direction (albeit at a relatively instantaneous point in time) it would yield a palaeomagnetic pole (24°S , 166°E) which lies about half way along the Grenville polar track (Fig. 4). Remanence B may thus have been acquired during the cooling stage of Grenville metamorphism. This interpretation, if correct, would suggest that the Grenville Province has

always remained part of North America, and furthermore, that Keweenaw igneous activity preceded acquisition of stable Grenville remanences. An alternative explanation for the B remanence is that it is much younger than the Grenville Province, perhaps about 700 Myr old, and represents a southerly extension of polar track 18 of Irving *et al.*¹⁵ (Fig. 4).

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- ¹ White, W. S., *US Geol. Surv. Prof. Paper*, 524E (1966).
- ² Halls, H. C., *Am. Geophys. Un. Monogr.*, 10, 3 (1966).
- ³ Dietz, R. S., *J. Geol.*, 67, 496 (1959).
- ⁴ French, B. M., *Bull. Volcanol.*, 34, 466 (1970).
- ⁵ Robertson, P. B., and Grieve, R. A. F., *Geophys. J. R. astr. Soc. Canada* (in the press).
- ⁶ Sage, R. P., *Misc. Paper No. 59*, 80 (Ontario Div. Mines, 1974).
- ⁷ Arfken, G., *Mathematical methods for Physicists* (Academic, New York, 1970).
- ⁸ Books, K. G., *US Geol. Surv. Prof. Paper*, 600D, (1968).
- ⁹ Palmer, H. C., *Can. J. Earth Sci.*, 7, 1410 (1970).
- ¹⁰ Halls, H. C., *Can. J. Earth Sci.*, 11, 1200 (1974).
- ¹¹ Pohl, J., and Soffel, H., *Z. Geophys.*, 37, 857 (1971).
- ¹² Currie, K. L., and Larochelle, A., *Earth planet. Sci. Lett.*, 6, 309 (1969).
- ¹³ Larochelle, A., and Currie, K. L., *J. geophys. Res.*, 72, 4163 (1967).
- ¹⁴ Robertson, W. A., *Can. J. Earth Sci.*, 4, 641 (1967).
- ¹⁵ Irving, E., Emslie, R. F., and Ueno, H., *J. geophys. Res.*, 79, 5491 (1974).
- ¹⁶ Buchan, K. L., and Dunlop, D. J., *Nature phys. Sci.*, 246, 28 (1973).
- ¹⁷ Fahrig, W. F., Christie, K. W., and Schwarz, E. J., *Can. J. Earth Sci.*, 11, 18 (1974).

Alkyl substituted cyclic ethers in 2,300 Myr old Transvaal algal stromatolite

Two cyclic ethers, 2-*n*-propyl-3-methyltetrahydrofuran and 2-*n*-propyltetrahydropyran, have been identified for the first time from the insoluble polymer-like kerogen in a Precambrian rock by ozonolysis, gas chromatography and mass spectrometry. These compounds could prove to be the oldest indigenous biochemical fossils. This same carbonate stromatolite contains abundant and well preserved fossil coccolith and filamentous blue-green algae, some of which show the first known evidence of the diversification of cells¹. Many of the filamentous algae in this and other Transvaal stromatolites contain specialised cells, akinite and heterocyst-like structures^{1,2}. This sample was obtained 750 m stratigraphically above the base of the Transvaal Sequence from an outcrop approximately 315 km north-east of Johannesburg, South Africa³. Using isotope dating^{4,5} the age of this sample has been estimated to be ~2,300 Myr (D. A. Pretorius, personal communication).

To remove all possible contamination before analysis the surface layer of the sample was removed with a high speed drill, sonicated in triple distilled water, and finally pulverised and extracted consecutively with distilled benzene-methanol, methanol and water. This removes non-indigenous soluble substances which can invade even dense rocks⁶⁻⁸. Next, the carbonate rock was dissolved in distilled HCl, and the residue was re-extracted with the same organic solvents as before. This residue was then ozonised, treated with 30% H₂O₂, and extracted with freshly distilled diethyl ether⁹. A complete blank experiment using ignited basalt powder and the evaporation residue of 1,000 ml of the diethyl ether did not contain any cyclic ethers or other organic compounds.

The ether extracts were analysed by combined gas chromatography-mass spectrometry. A 100 foot × 0.02 inch internal diameter OS-138 (support coated open tubular, SCOT) column was the most effective for separating the two major peaks. Four other SCOT columns, three 150 foot × 0.02 inch and one 100 foot × 0.02 inch, Apiezon-L, SE-30,

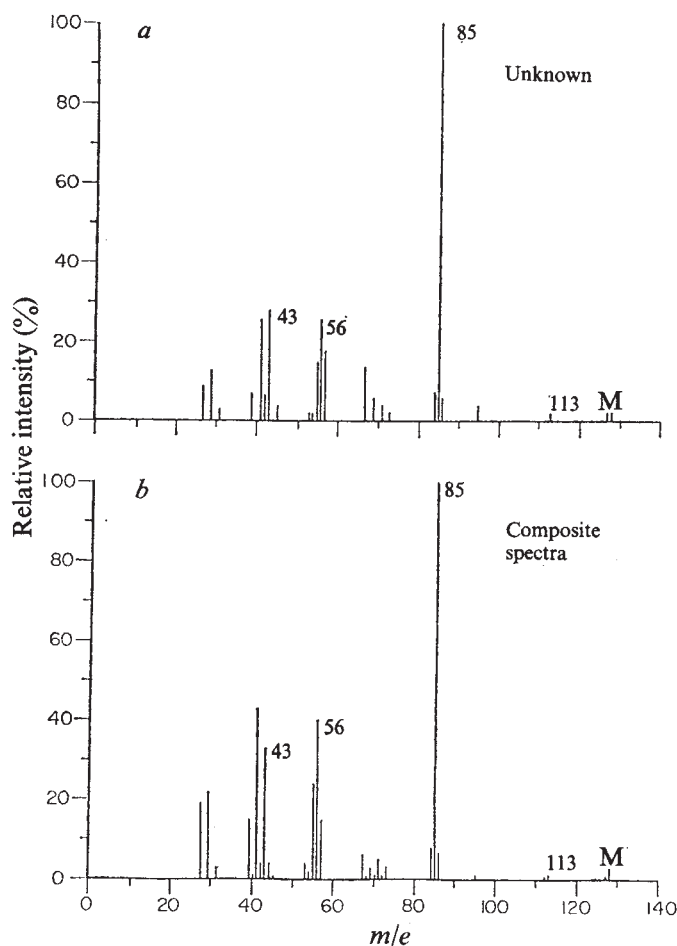


Fig. 1 *a*, Mass spectrometric fragmentation pattern of the first of two prominent gas chromatographic peaks of the ether extract of ozonised Transvaal stromatolite kerogen. This peak is overlapped and contains 25% of the adjacent, second prominent peak. 70 eV. *b*, Composite mass spectrum consisting of 75% 2-*n*-propyl-3-methyltetrahydrofuran and 25% 2-*n*-propyltetrahydropyran. 70 eV. M, *m/e* = 128.

Dexsil-300 and OV-17, respectively, were evaluated but found to be less effective. Two major components were eluted and partially separated from the ether extract by the OS-138 column. A single focusing Hitachi-RMU-6E mass spectrometer provided rapid scan mass spectra for each component. The mass spectrum of the first major eluate is shown in Fig. 1*a*. In Fig. 1*b* we show a composite mass spectrum consisting of a mixture of 75% 2-*n*-propyl-3-methyltetrahydrofuran and 25% 2-*n*-propyltetrahydropyran standards, because it was established by extrapolation that the first major gas chromatographic peak (corresponding to 2-*n*-propyl-3-methyltetrahydrofuran) overlapped the second major peak by 25%.

This composite mass spectrum closely resembles the mass spectrum of the first major gas chromatographic peak. The mass spectrum of the second peak also corresponds to a composite spectrum of the same standards containing considerably more 2-*n*-propyltetrahydropyran.

Figures 2*a* and 2*b* show the mass spectra of the 2-*n*-propyl-3-methyltetrahydrofuran (1:1 mixture *cis* and *trans* isomers) and 2-*n*-propyltetrahydropyran standards, respectively. Both the unknown components and the standards have identical parent ions (*m/e* = 128) and base peaks (*m/e* = 85). α -Cleavage of the propyl substituent on tetrahydropyran or methyltetrahydrofuran gives rise to the characteristic *m/e* = 85 base peak¹⁰. High resolution mass spectrometry of the unfractionated diethyl ether extract

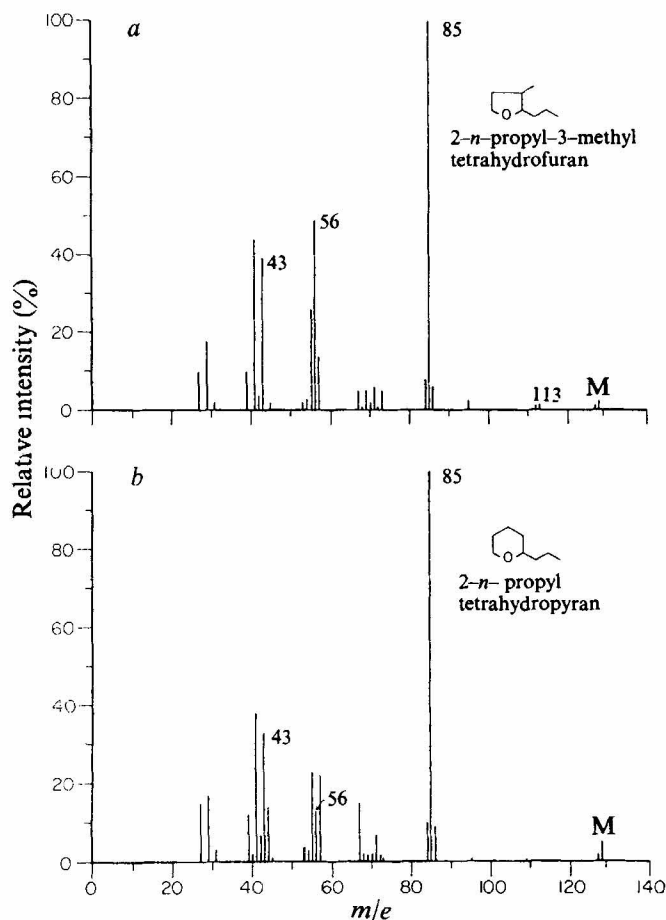


Fig. 2 Mass spectrometric fragmentation patterns of: *a*, 2-*n*-propyl-3-methyltetrahydrofuran; *b*, 2-*n*-propyltetrahydropyran standards. 70 eV.

showed many ions, but only one for the composition $C_8H_{16}O$, which is the parent ion (M) of methyl, propyltetrahydrofuran and propyltetrahydropyran ($m/e=128.11869$).

The M-15 ion (loss of CH_3) was established, $m/e=113.09660$, corresponding to $C_7H_{13}O$. The base peak gave two compositions, $m/e=85.06570$ and 85.02839 , corresponding to C_5H_9O and $C_4H_7O_2$, respectively. The latter, unrelated to the methyltetrahydrofuran and tetrahydropyran fragment ions, was much less abundant than C_5H_9O . Thus, 2-*n*-propyl-3-methyltetrahydrofuran and 2-*n*-propyltetrahydropyran were identified by mass spectrometry in the ether extract of the ozonised kerogen from the algal stromatolite.

The identification raises three questions: are the cyclic ethers ozonolysis artefacts; are they part of kerogen since they do not contain the characteristic carboxyl groups⁹ of ozonolysis degradation products; and what is their origin? The first possibility is unlikely because these cyclic ethers are not known to be ozonolysis artefacts arising from alkanes during the relatively mild procedures applied¹¹ (G. A. Hamilton, personal communication). Furthermore, ozonolysis of double bonds will result in *cis*-cycloaddition of O_3 causing bond rupture. The second question is more difficult to answer but the two cyclic ethers, liquid at room temperature, should have been removed by solvent extractions if they were not trapped before and after the dissolution of the rock. Perhaps they were trapped mechanically and/or held by weak bonding inside the kerogen matrix.

Accounting for the origin of these cyclic ethers forces one to propose a hypothesis which should be geologically, biologically and chemically plausible because these compounds occur in a rock built by¹² and containing abundant

filamentous blue-green algae. The major component of some of these specialised cells are carbohydrates (on a dry weight basis) yet the cyclic ethers do not have carbon skeletons identifiable with common biochemicals. (Most coal degradation products do not have biochemical carbon skeletons either.) One of the many possible mechanisms for their biological origin is suggested: acid converts hexoses, such as glucose, to 5-hydroxymethylfurfural¹³. Acid catalysed condensation with pyruvic or oxalacetic acids, important biochemicals associated with the metabolism of carbohydrates, could yield 2-(3-keto-3-carboxylprop-1-ene)-5-hydroxymethylfuran^{14,15}. During diagenesis, reduction of keto and olefinic groups may occur by hydrogenation accompanied by decarboxylation¹⁶. The 5-hydroxymethyl group on the resultant 2-*n*-propyltetrahydrofuran moiety could form a primary carbonium ion in the presence of alumina gel (and certain clays) with subsequent rearrangement yielding the more stable secondary carbonium ion leading to 2-*n*-propyl-4,5-dihydropyran¹⁷. Further reduction could produce 2-*n*-propyltetrahydropyran.

Catalytic O_2 oxidation (perhaps from algal photosynthetic O_2) or microbial oxidation of the 5-hydroxymethylfurfural could yield 5-carboxylfurfural. Condensation with pyruvic or oxalacetic acid would yield 2-(3-keto-3-carboxylprop-1-ene)-5-carboxylfuran. (Thus, the 2- and 5-positions on the furan ring are blocked, and the 4-position is deactivated by the carboxyl group.) Formaldehyde, an oxidation product of methanol and breakdown product of certain amino acids, in the presence of HCl (one component¹⁸ of known, nearby, simultaneous volcanic events³) may provide the methyl group in the 3-position¹⁹. Subsequent reduction and decarboxylation^{16,20} during diagenesis could yield the 2-*n*-propyl-3-methyltetrahydrofuran.

The suggested pathways for the evolution of both cyclic ethers first require a biochemical and subsequently a geological environment. The precursors and, therefore, the products of these suggested reaction mechanisms would be compatible with the biochemistry of the modern blue-green alga, *Raphidiopsis*. This alga is analogous¹ to the oldest known complex life forms found in this same sample.

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- Nagy, L. A., *Science*, **183**, 514 (1974).
- MacGregor, I. M., Truswell, J. R., and Eriksson, K. A., *Nature*, **247**, 538 (1974).
- Button, A., *J. sedim. Petrol.*, **43**, 160 (1973).
- Van Niekerk, C. B., and Burger, A. J., *Ann. Geol. Surv. S. Afr.*, **3**, 75 (1964).
- Davis, R. D., Allsopp, H. L., Erlank, A. J., and Manton, W. I., *Spec. Publ. geol. Soc. S. Afr.*, **1**, 763 (1970).
- Abelson, P. H., and Hare, P. E., *Yb. Carnegie Instn Wash.*, **67**, 208 (1969).
- Nagy, B., *Geochim. cosmochim. Acta*, **34**, 525 (1970).
- Schopf, J. W., *Biol. Rev.*, **45**, 319 (1970).
- Bitz, M. C., and Nagy, B., *Proc. natn. Acad. Sci. U.S.A.*, **56**, 1383 (1966).
- Biemann, K., *Mass Spectrometry*, 99 (McGraw-Hill, New York, 1962).
- Hamilton, G. A., Ribner, B. S., and Helman, T. M., *Adv. Chem. Ser.*, **77**, 15 (1968).
- Schopf, J. W., *Ann. Revs Earth planet. Sci.* (in the press).
- Katritzky, A. R., and Lagowski, J. M., *The Principles of Heterocyclic Chemistry*, 104 (Academic, New York, 1968).
- Houze, H. O., *Modern Synthetic Reactions*, 217 (Benjamin, New York, 1965).
- Johnson, J. R., and Hager, F. D., *Org. Synth.*, **1**, 351 (1944).
- Parker, P. L., in *Organic Geochemistry*, 363 and 366 (Springer-Verlag, New York, 1969).
- Acheson, R. M., *An Introduction to the Chemistry of Heterocyclic Compounds*, 104 (Wiley, New York, 1967).
- Krauskopf, K. B., *Introduction to Geochemistry*, 461 (McGraw-Hill, New York, 1967).
- Walker, J. F., *Formaldehyde*, 451 (Reinhold, New York, 1964).
- Katritzky, A. R., and Lagowski, J. M., *The Principles of Heterocyclic Chemistry*, 123 (Academic, New York, 1968).

Seasonal high and low density bands in reef coral skeletons

THE skeletal aragonite deposited by scleractinian reef corals is characterised by alternating bands of high and low density carbonate that are concordant with the growth surface of the colony. These density variations, which are readily observed by X-radiographic examination of 5–10 mm thick sections of the corallum cut parallel to the axis of growth, are annual in periodicity, such that one high density band plus one adjacent low density band represent the skeletal increment formed over one calendar year^{1–4}. Skeletal X radiography is a powerful tool for studying both modern and fossil corals, for in addition to providing a wealth of growth rate data from specimens that grew undisturbed for many years in their natural habitat on the reef, valuable information about the coral itself and about the environment in which the coral lived can be obtained. As Buddemeier⁵ points out, density banding in reef corals can not only yield physiological and ecological information about these marine organisms, but also help to unlock the record of long term environmental conditions that is embodied in a coral skeleton. Reef corals, for example, are effective samplers of certain important radionuclides⁶.

Although a density band pair clearly corresponds to an annual increment of carbonate deposition, it has not yet been determined when during a year a growth band of particular density actually forms. Thus until a quantitative relationship between density patterns and environmental parameters is established either on an empirical basis or through an improved understanding of coral physiology, the full potential of reef corals in reconstructing climatic history of the recent past cannot be realised⁵. Different attempts to determine when, or during what season, a high or a low density band forms have led to remarkably contradictory conclusions. Some investigators^{4,6–8} suggest that the high density band is associated with seasonal low water temperature (such as during Northern Hemisphere winter), whereas others^{2,9,10} report that high skeletal density is correlated with periods of high water temperature.

The techniques used so far to resolve this problem involve either: incorporating a fiducial time marker in the skeleton of a living coral (perhaps by *in situ* staining with alizarin red S or similar dyes at a particular time of the year), enabling the animal to continue growing for an extended period, and finally examining the skeleton by X radiography⁷; or correlating the density of the outermost (last formed) growth band with the time of collection of living corals (and thus with environmental parameters such as water temperature and illumination levels) using a large number of specimens removed from both Northern and Southern Hemisphere reef localities on known dates^{9,10}.

A different approach, described here, is based on the fact that the oxygen isotope ratio of the skeletal carbonate is strongly temperature dependent^{11,12}, larger $^{18}\text{O}/^{16}\text{O}$ ratios being associated with lower water temperatures. Isotope ratios are expressed in conventional notation as $\delta^{18}\text{O} = 1,000 (R_x/R_s - 1)$, in ‰, where R is the $^{18}\text{O}/^{16}\text{O}$ ratio; x and s refer to sample and standard (PDB CO_2) respectively. For $\delta^{13}\text{C}$, R in this above equation corresponds to the $^{13}\text{C}/^{12}\text{C}$ ratio. Using the subscripts c and w to indicate skeletal carbonate derived from coral and seawater respectively, $\delta^{18}\text{O}_c$ is determined by three factors: temperature (T) of deposition; $\delta^{18}\text{O}_w$; and the relative amounts (A) of carbonate oxygen obtained from two different sources, bicarbonate dissolved in seawater, and metabolic carbon dioxide¹³. The effect of $\delta^{18}\text{O}_w$ can be eliminated using corals from localities where seasonal changes in $\delta^{18}\text{O}_w$ are almost entirely absent. Thus if variation in $\delta^{18}\text{O}_c$ due to variation in A can be removed, the remaining variation in $\delta^{18}\text{O}_c$ can be attributed to variation in T . In this way it is possible to relate $\delta^{18}\text{O}_c$ to density differences between growth bands in a coral skeleton, and thus to relate such density differences to T .

Because $\delta^{13}\text{C}_c$ and $\delta^{18}\text{O}_c$ are positively (directly) correlated as a result of simple mixing of two components (HCO_3^- enriched in ^{13}C and ^{18}O ; metabolic CO_2 enriched in ^{12}C and ^{16}O)¹³, it is necessary to measure only three variables simultaneously in order to establish a unique relationship between T and skeletal density (D). These variables are: (I) $\delta^{13}\text{C}_c$; (II) $\delta^{18}\text{O}_c$; and (III) D . From a set of such data adequate in the statistical sense, the influence of A can be eliminated by partial correlation analysis¹⁴ by calculation of $r_{II\ III\ .\ I}$, that is, the coefficient of correlation between variables II and III independent of variable I.

For an investigation of this sort, it is desirable to examine corals from localities characterised by rather large seasonal changes in water temperature, as $\delta^{18}\text{O}_c$ differences between high and low density bands will be correspondingly large, and experimental error involved in eliminating the effect of A will be minimised. This qualification, however, must be balanced by the fact that the thickness (width) of the density bands decreases with decreasing mean annual water temperature^{15,16}, that localities with large seasonal changes in water temperature also have relatively low values for mean annual water temperature, and that a finite quantity of skeletal carbonate is required to carry out an isotopic analysis.

After X-radiographic examination of 1,488 specimens of modern reef corals representing 31 reef localities widely distributed over the Indo-Pacific and Caribbean regions, a large colony of *Porites lutea* from Heron Island, Australia (23°S 152°E), was selected for detailed study. At this offshore site on the southern Great Barrier Reef, winter to summer monthly water temperature ranges between 20.9 and 26.5 °C. An X radiograph of this specimen was used as a guide in locating the position of 45 subsamples removed from a section of corallum with a vanadium-steel cylindrical cutter 3 mm in diameter. After drilling the 45 holes, the section was again X radiographed to confirm the precise location of the subsamples with respect to the density bands. Isotope ratios were determined by high-precision mass spectrometry^{17,18} using analytical techniques providing reproducibility of 0.04‰ (standard deviation) for $\delta^{13}\text{C}$, and 0.05‰ for $\delta^{18}\text{O}$. D is expressed as the percentage of the high density band included in the subsample; as the diameter of the cutting tool slightly exceeds the width of the high density band, values of D were restricted to the range 0–75%.

Correlation coefficients calculated from these data for the three variables defined above are: $r_{I\ II} = 0.5860$; $r_{I\ III} = 0.1908$; $r_{II\ III} = -0.1259$. The partial correlation coefficient¹⁴ $r_{II\ III\ .\ I}$, which expresses the degree of correlation between $\delta^{18}\text{O}_c$ and D independent of $\delta^{13}\text{C}_c$, is -0.2989 . With 42 degrees of freedom, this value of r is statistically significant at the 5% level. As indicated by the negative sign, the correlation between $\delta^{18}\text{O}_c$ and D is negative (inverse), that is, greater skeletal density is associated with lower $^{18}\text{O}_c$ content and thus with higher water temperature. On this basis, we conclude that the high density band in a reef coral skeleton forms during that time of the year when water temperatures are relatively high. This supports our earlier conclusion from a study of the density of the outermost growth band in 1,488 X-radiographed coral specimens that were collected on known dates^{9,10}.

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¹ Knutson, D. K., Buddemeier, R. W., and Smith, S. V., *Science*, **177**, 270–272 (1972).

² Macintyre, I. G., and Smith, S. V., in *Proc. second Int. Symp. Coral Reefs, Australia*, **2**, 277–287 (1974).

- ³ Moore, W. S., and Krishnaswami, S., *ibid.*, 2, 269–276 (1974).
- ⁴ Dodge, R. E., and Thomson, J., *Earth planet. Sci. Lett.*, 23, 313–322 (1974).
- ⁵ Buddemeier, R. W., in *Proc. second Int. Symp. Coral Reefs, Australia*, 2, 259–267 (1974).
- ⁶ Knutson, D. K., and Buddemeier, R. W., *Int. Atom. Energy Agency (Vienna) Publ. SM-158/49*, 735–746 (1973).
- ⁷ Buddemeier, R. W., Maragos, J. E., and Knutson, D. K., *J. exp. Mar. Biol. Ecol.*, 14, 179–200 (1974).
- ⁸ Buddemeier, R. W., and Kinzie, R. A., *Proc. Newcastle Interdisciplinary Conf. Biological Clocks and Changes in the Earth's Rotation* (Wiley, in the press).
- ⁹ Weber, J. N., and White, E. W., *Geol. Soc. Am. Abstr. with Programs*, 6, (7) 1003 (1974).
- ¹⁰ Weber, J. N., and White, E. W., *J. Paleobiol.*, 1 (in the press).
- ¹¹ Weber, J. N., and Woodhead, P. M. J., *J. geophys. Res.*, 77, 463–473 (1972).
- ¹² Weber, J. N., and Woodhead, P. M. J., *Mar. Biol.*, 15, 293–297 (1972).
- ¹³ Weber, J. N., and Woodhead, P. M. J., *Chem. Geol.*, 6, 93–117 (1970).
- ¹⁴ Snedecor, G. W., *Statistical Methods*, 429–432 (Iowa State University Press, 1956).
- ¹⁵ Weber, J. N., and White, E. W., *Mar. Biol.*, 26, 353–359 (1974).
- ¹⁶ Weber, J. N., and White, E. W., *Proc. seventh Caribbean Geol. Conf., Guadeloupe* (in the press).
- ¹⁷ Weber, J. N., and Raup, D. M., *Geochim. cosmochim. Acta*, 30, 681–736 (1966).
- ¹⁸ Deines, P., *Int. J. Mass Spectrom. Ion Phys.*, 4, 283–295 (1970).

Possible occurrence of marsupial bones in Cretaceous eutherian mammals

MARSUPIAL bones (epipubes, prepubes) occur in Marsupialia, Monotremata and have also been found in Cretaceous Multituberculata¹. Gregory and Camp² and Romer³ supposed that prepubic cartilages existed in therapsids. Fourie^{4,5} described a tritylodontid skeleton in which these bones were preserved. Marsupial bones have not been found in eutherian mammals unless one accepts Jellison's⁶ idea that the baculum is homologous to the epipubic bones. The earliest known eutherian postcranial skeletons come from the Djadokhta Formation (?Santonian) and Barun Goyot Formation (?middle Campanian) in the Gobi Desert, Mongolia^{7,8} (and are now in the Zaklad Paleozoologii, Warsaw). The most complete are the skeletons of *Zalambdalestes* and *Barunlestes*⁸, both assigned to the family Zalambdalestidae. Study of the structure of their dentition and skulls leaves no doubts that the Zalambdalestidae are members of the Eutheria.

A partial skeleton of *Barunlestes butleri* Kielan-Jaworowska, from the Barun Goyot Formation, Khulsan locality in the Gobi Desert, Mongolia (No. MgM-1/77) includes the right os coxae, the ventral margin of which is not complete. The anterior margin of the acetabular branch of the pubic bone has a structure unusual for eutherian mammals. On the lower part of this margin a triangular, concave surface is arranged at an obtuse angle to the outer surface of the pubic bone. The triangular concavity is elongated dorsoventrally, pointed downwards, with a tubercle on its upper anterior corner and a medial longitudinal groove. The concavity is separated from the outer surface of the pubis by a distinct ridge, more prominent dorsally than ventrally. This triangular fossa is interpreted to be an articular surface for a marsupial bone.

It is not clear from the description and photographs presented by Fourie^{4,5} whether a fossa is present on the anterior margin of the pubis in Tritylodontidae. In Cretaceous Multituberculata the articular surface is absent from the anterior margin of the pubis. In Monotremata (Fig. 1a) there is a distinct, oval fossa on the lower part of the anterior margin of the pubis, which articulates with the thickened posterior border of the marsupial bone. Anterodorsal to the fossa there is sometimes a small apophysis, which articulates with the posterodorsal apophysis of the marsupial bone. The shape of the fossa, which is very distinctly outlined and deep, is different from that in *Barunlestes* (Fig. 1b). Postcranial skeletons of Cretaceous Marsupialia have not been recognised and it is as yet impossible to conclude whether such a fossa occurred in early marsupials. In modern marsupials the articular area for the marsupial bone strongly varies in shape. In most marsupials known to me there is no fossa on the anterior edge of the pubis, and in some families (for example, Didelphidae and Vombatidae) instead of a fossa there is

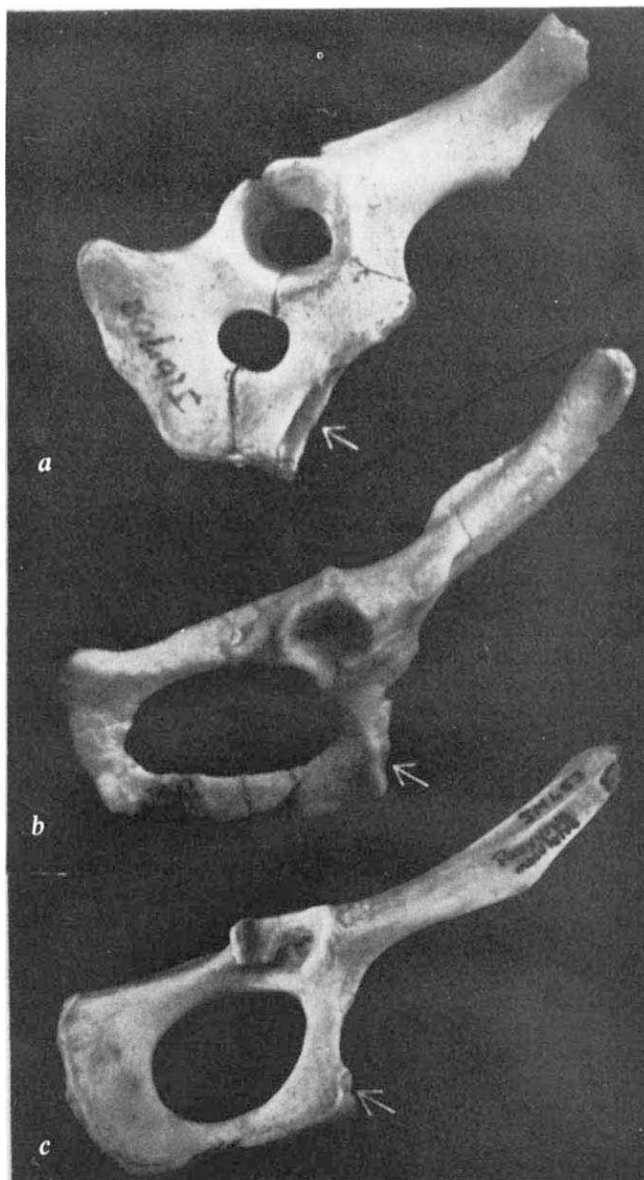


Fig. 1 Right os coxae. Arrows denote the articular surfaces on the pubis. a, *Tachyglossus* (×1.4); b, *Barunlestes* (×3); c, *Dasyurus* (×1).

an oval condyle. In the Dasyuridae (for example, *Dasyurus*, Fig. 1c) at the lower part of the anterior edge of the pubis, however, there is a small, shallow, irregular fossa, comparatively shorter than in *Barunlestes*. These comparisons suggest that marsupial bones may have occurred in the Cretaceous eutherian *Zalambdalestes*.

The presence or absence of the marsupial bones in the extinct orders Triconodonta, Docodonta, Symmetrodonta and Pantotheria cannot as yet be determined because the postcranial skeletons have either not been found or not yet described (Morganucodontidae). It is possible, however, that marsupial bones were present in all these early mammals. Disappearance of marsupial bones in the Tertiary Eutheria may be connected with the gradual evolution of prolonged internal gestation period.

The pelvis in mammals is variously shaped according to the mode of reproduction. It is widely open posteriorly and the ischial arc is U-shaped in Monotremata (Fig. 2a), possibly in connection with oviparity. The ischial arc is V-shaped in Marsupialia (Fig. 2b and c), but the angle at which the ischia meet may vary. The shape of the ischial

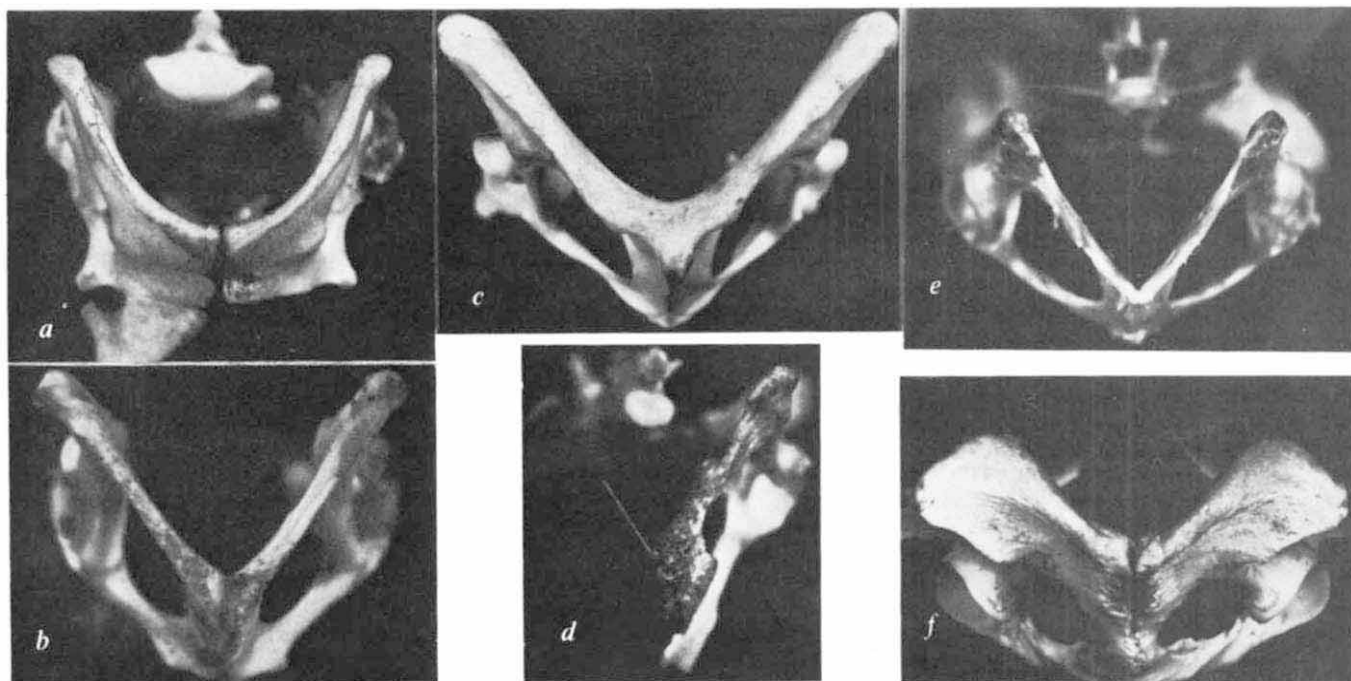


Fig. 2 Ischial arc in posteroventral view in: a, *Tachyglossus* ($\times 1.4$); b, *Didelphis* ($\times 1.75$); c, *Macropus* ($\times 0.7$); d, *Barunlestes* ($\times 3.5$); e, *Tupaia* ($\times 2.8$); f, *Equus* ($\times 0.28$).

arc in modern eutherian mammals varies from comparatively narrow and V-shaped in nidicoles (Fig. 2e) up to a widely open condition, forming an obtuse angle in advanced nidifuges (Fig. 2f). The differences in shape of the ischial arc between marsupials and nidicolous eutherians are not very great, and in some marsupials (for example, *Macropus*) the ischial arc is more widely open dorsally than in certain nidicolous eutherians (for example, *Tupaia*). In spite of this, one can state that in viviparous mammals in general, the ischial arc widens with prolonged periods of gestation. It follows that the shape of the ischial arc together with other characters (such as the occurrence of marsupial bones) may provide some indication of the mode of reproduction in fossil mammals. In *Barunlestes* the ischial arc is V-shaped and very narrow (Fig. 2d). Together with the presence of the marsupial bones this may indicate a short gestation period.

Lillegraven^{9,10}, partially on the basis of embryological evidence of Müller¹¹, suggested that the neonate in common ancestors of marsupials and placentals was born free of egg membranes, in an immature, marsupial-like state. It seems possible that Cretaceous eutherian mammals retained this primitive condition and were born in a much more nidicolous state than most modern eutherian mammals. More detailed discussion on the marsupial characters in the skeletons of Cretaceous Eutheria and their phylogenetic implications will be published later.

I thank Dr M. Archer (Queensland Museum, Brisbane) for the loan of marsupial skeletons and for the most valuable information on the structure of the pelvis in Australian marsupials; Professors A. W. Clemens (University of California, Berkeley) and J. A. Lillegraven (California State University, San Diego) for comments and discussion.

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⁷ Kielan-Jaworowska, Z., *Pal. Polonica*, 19, 1970 (1969).

⁸ Kielan-Jaworowska, Z., *Pal. Polonica* (in the press).

⁹ Lillegraven, J. A., *Paleont. Contr. Univ. Kansas*, Art. 50, (Vertebrata 12) (1969).

¹⁰ Lillegraven, J. A., *Evolution* (in the press).

¹¹ Müller, F., *Rev. Suisse Zool.*, 79, (1972).

Comparative effects of atmospheric pollution and cigarette smoking on carboxyhaemoglobin levels in man

ATMOSPHERIC pollution by carbon monoxide (CO), mainly derived from the motor-car, has given rise to worldwide concern in recent years. In an attempt to limit this form of pollution legislation has been introduced, both in the US¹ and Britain², to control the level of CO emitted by the exhaust of the internal combustion engine. The cost of such measures would be high, both in terms of engine modification and the inevitably increased fuel consumption. The question arises: What would be the probable effect of such measures on man?

When inhaled, CO combines with haemoglobin in the red cells to form the stable compound carboxyhaemoglobin (COHb) in a quantitative manner, and although the affinity of haemoglobin for CO is over 200 times greater than its affinity for oxygen the rate of combination is markedly slower³. The main physiological effects of COHb are twofold; an absolute decrease in haemoglobin available for oxygen carriage and interference with oxygen transport by the remaining available haemoglobin. These effects combine to decrease the oxygen supply to the tissues.

Atmospheric concentrations of CO vary widely throughout the world, ranging from extremely low concentrations in the Central Pacific⁴ to over 100 p.p.m. in urban highways in peak conditions of traffic flow⁵. The average concentration in a major city street, however, seems to be about 10–30 p.p.m. and this applies all over Europe⁶.

One place near to Britain which has low atmospheric concentrations of CO is the Channel Island of Sark. This is a relatively isolated community where motor cars are forbidden by the Dame of Sark. The only mechanical means of transport on the Island is by diesel powered tractor, which emit only low concentrations of CO. Twenty spot estimations of island air over a 6 d period always revealed concentrations of less than 1 p.p.m. CO. Blood samples taken at the same time were

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¹ Kielan-Jaworowska, Z., *Nature*, 222, 5198 (1969).

² Gregory, W. K., and Camp, C. L., *Bull. Am. Mus. Nat. Hist.*, 38, 447 (1918).

³ Romer, A. S., *Osteology of Reptiles* (University of Chicago Press, 1956).

⁴ Fourie, S., *Natur. Nas. Mus.*, 2, 7 (1962).

⁵ Fourie, S., *Nature*, 198, 201 (1963).

⁶ Jellison, W. L., *J. Mammal.*, 26, 146 (1945).

estimated for COHb by the method of Commins and Lawther^{7,8}; 78 subjects of both sexes who did not smoke had a mean COHb of 0.68% with a range of 0.2–2.6% (Fig. 1a). Female subjects had a mean COHb of 0.70% compared with male subjects whose mean level was lower, 0.61%. This difference is not significant.

In the City of London, although street concentrations of CO are much higher than those in Sark most people are exposed to such an atmosphere for a relatively short period of time. Concentrations of CO in the atmosphere breathed by most people during the greater part of their working lives are in fact much lower than those in the street. By far the greatest proportion of the population commute from suburban areas where atmospheric concentrations of CO are only 1–2 p.p.m. away from streets (unpublished data).

In the Outpatient Department of St Bartholomew's Hospital (where smoking is forbidden), the ambient CO concentrations ranged between 2 and 4 p.p.m. The resultant mean COHb level in 120 non-smokers was 0.97% with a range of 0.2–2.5% (Fig. 1b). In an adjacent City office (where smoking was permitted) the ambient concentration was slightly higher,

Fig. 1 COHb levels in three populations of non-smokers. *a*, Island of Sark (the two highest levels were both from women who may have been exposed to a defective butane gas burner before sampling). Mean COHb 0.68% atmospheric CO <1 p.p.m. *b*, City of London, where smoking was not permitted. Mean COHb 0.97%; atmospheric CO 2–4 p.p.m. *c*, City of London, where smoking was permitted. Mean COHb 1.12%; atmospheric CO 3–8 p.p.m. Groups *b* and *c* were sampled between 1400 and 1600.

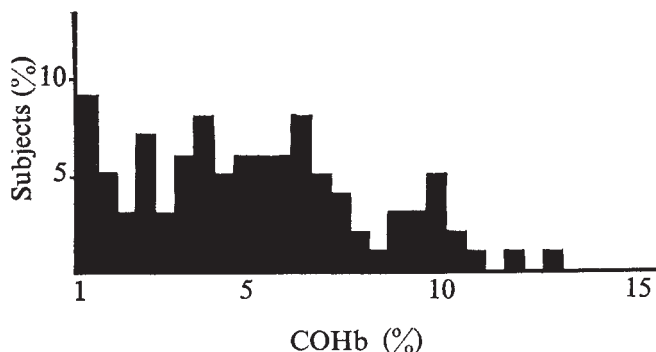
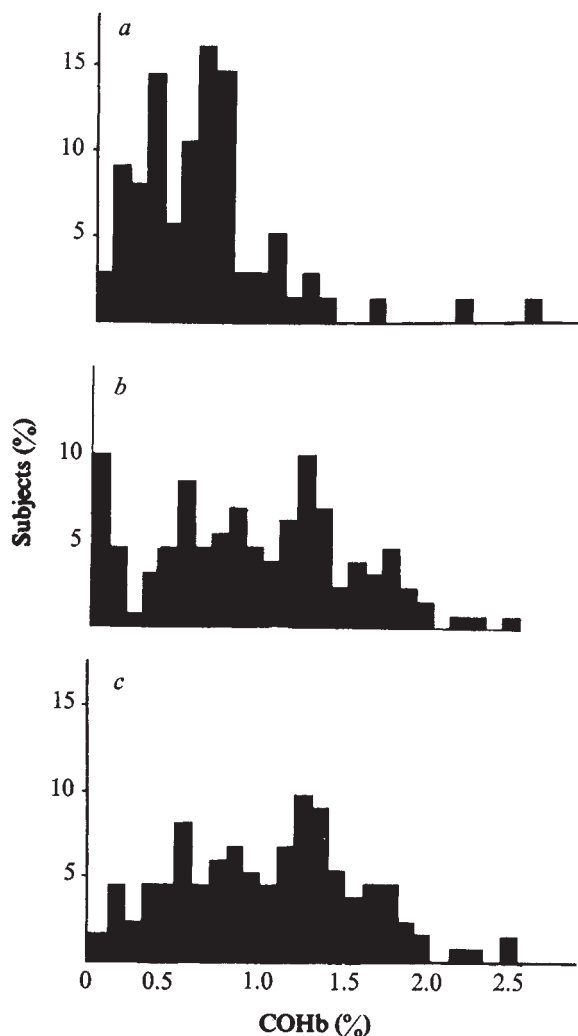


Fig. 2 COHb levels in 100 city office workers who smoked. All samples were taken between 1400 and 1600.

3–8 p.p.m. CO. COHb levels in 100 office workers (who were non-smokers) were also slightly higher, a mean of 1.12% with a range of 0.1–2.7% (Fig. 1c). COHb levels were also estimated at the same time for 100 office workers in the same environment who were regular cigarette smokers. Their mean COHb was 5.5% with a range of 2.2–13.0% (Fig. 2)—very much higher than any of the previous groups.

The resting COHb level of a non-smoker, not exposed to atmospheric carbon monoxide is not zero but about 0.7% (ref. 9). This is derived from the endogenous production of CO which is produced in the body by the normal breakdown of haem-containing compounds, mainly haemoglobin. When man is continually exposed to atmospheres similar to those breathed by most workers in the City of London but not contaminated by cigarette smoke his COHb rises on average by about 0.3%. If smoking is permitted in this atmosphere slightly higher concentrations of CO are found and in turn, slightly higher COHb concentrations in those who breathe it—the increase being about half that resulting from the urban environment alone. This increase is almost certainly caused by 'passive smoking'—the inhalation of side-stream and exhaled main-stream smoke from fellow workers who actively smoke¹⁰. Main-stream cigarette smoke contains up to 5% CO (ref. 11), cigar smoke perhaps even twice as much. Active inhalation of this produces levels of COHb which are considerably higher than those found in any other group of subjects who work in the City of London. Even traffic policemen, who spend long periods of time in busy city streets, have significantly higher COHb levels than non-smoking policemen performing similar duties¹². It is unlikely that COHb levels similar to those found in our non-smoking subjects, could be shown to be harmful to health. Levels similar to those commonly found in cigarette smokers, however, are probably responsible for many disease processes particularly those associated with the cardiovascular system^{13,14}.

To conform with the present European standard of exhaust emission control, the cost of the necessary carburettor modification is relatively modest. To conform with projected US standards the overall retail cost of a vehicle would be increased by about 9%. Losses of fuel economy would be of the order of 15% (ref. 15). The annual cost of such legislation could well be many hundreds of millions of pounds. In addition there is a world shortage of fossil fuels. Replacement of London's atmosphere by one similar to that of Sark might be expected to reduce the mean COHb level of its inhabitants by 0.3%. In practice the effects would be even less! Even the most severe controls cannot, at present, remove all the emitted CO from a vehicle exhaust. Furthermore the many other sources of urban atmospheric CO will not be affected. Even if a reduction is achieved, it will only affect a relatively small proportion of the population—dwellers in large towns who do not smoke. The effects on smokers could not even be measured—and 70% of adult males smoke¹⁶.

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- ¹ Federal Register, 37, No. 221, part 2 (1972).
- ² Motor Vehicles (Construction and Use), Regulation 33A (HMSO, 1973).
- ³ Holland, R. A. B., *Ann. N.Y. Acad. Sci.*, 174, 154-171 (1970).
- ⁴ Robinson, E., and Robbins, R. C., *Ann. N.Y. Acad. Sci.*, 174, 89-95 (1970).
- ⁵ Waller, R. E., Commins, B. T., and Lawther, P. J., *Br. J. Industr. Med.*, 22, 128-138 (1965).
- ⁶ Chovin, P., and Truffert, L., *Proc. Health Effects of Carbon Monoxide Environmental Pollution, Luxembourg, 1973*, 151-194 (Office for Official Publication of European Communities, Luxembourg, 1974).
- ⁷ Commins, B. T., and Lawther, P. J., *Br. J. Industr. Med.*, 22, 139-144 (1965).
- ⁸ Lily, R. E. C., Cole, P. V., and Hawkins, L. H., *Br. J. Industr. Med.*, 29, 454-457 (1972).
- ⁹ Sjöstrand, T., *Scand. J. clin. Lab. Invest.*, 1, 201-214 (1949).
- ¹⁰ Haake, H. P., Baars, A., Frahm, B., Peters, H., and Schultz, Ch., *Int. Arch. Arbeits Med.*, 29, 323-339 (1972).
- ¹¹ Wynder, E. L., and Hoffman, D., *Tobacco and Tobacco Smoke, Studies in Experimental Carcinogenesis* (Academic, New York, 1967).
- ¹² Lawther, P. J., and Commins, B. T., *Ann. N.Y. Acad. Sci.*, 174, 135-147 (1970).
- ¹³ Astrup, P., *Br. med. J.*, iv, 447-452 (1972).
- ¹⁴ Wald, N., Haward, S., Smith, P. G., and Kjeldson, K., *Br. med. J.*, i, 761-765 (1973).
- ¹⁵ Anderson, J. G., *Automotive Design Engin.*, 17, 12-13 (1974).
- ¹⁶ Royal College of Physicians, *Smoking and Health Now* (Pitman Medical, London, 1971).

Heavy metal pollution and mineralisation of nitrogen in forest soils

LITER decomposition and soil enzymatic activity in forests surrounding metal-processing industries are influenced by high concentrations of deposited heavy metals¹⁻². Very little seems to be known about the effects of heavy metal pollution on nitrogen mineralisation rates in natural soils. Reports on nitrogen mineralisation and nitrification rates after addition of heavy metal salts to mineral soils are not quite unanimous³⁻⁶. There is evidence, however, that the ammonification rate of some vineyard soils is retarded by accumulated Cu⁷. I have determined the relationship between nitrogen mineralisation rate and the degree of Cu and Zn pollution in conifer forest soils, and found that soil nitrogen mineralisation is impaired by industrial metal pollution. I also conclude that a Cu and Zn concentration of three times the background level is sufficient to bring about a measurable disturbance of the nitrogen mineralisation rate.

I have studied the surroundings of Gusum (58°15'N, 16°33'E), a village (1,500 inhabitants) in south-eastern Sweden, where only one industry is located, a brass mill founded in 1661. Considerable amounts of Cu and Zn, but also some Pb and Cd, have been deposited in the surroundings². Cu and zinc concentrations of the organic topsoil (mor horizon) in sites adjacent to the mill are as high as 10,000-15,000 p.p.m. and 15,000-20,000 p.p.m. dry weight, respectively. Background concentrations (Cu: 12-20 p.p.m., Zn: 100-200 p.p.m.) are found 7-9 km from the village. The vegetation near the mill has been severely injured.

The vegetation in the Gusum area is mainly coniferous woodland, with predominantly acid siliceous soils (archaeal moraines), usually podzolic types with a well defined mor horizon. Exposed bedrock (granite and gneiss) is widespread. The area is sparsely inhabited and there are no other industries of importance. As insignificant amounts of other pollutants are emitted from the mill and the village, the influence of the deposited heavy metals on soil biological processes may be studied without severe interference from other pollutants, a rare circumstance in empirical studies on industrial ecology.

Field sampling was carried out from March 30-April 2, 1974, on the mor horizon on the northern side of spruces,

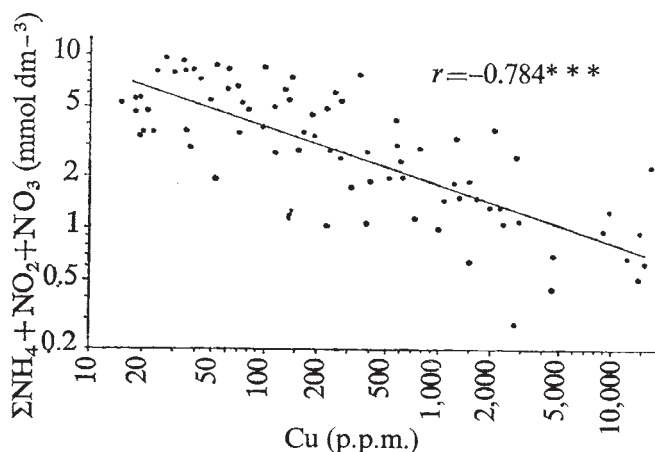
Table 1 Relationship between nitrogen mineralisation rate (y) and certain soil variables (x)

Total correlation, r_{xy}		
log Cu log y		-0.784***
log Zn log y		-0.734***
log Pb log y		-0.696***
log Cd log y		-0.669***
pH _{KCl} log y		0.087
Partial correlation, $r_{x1y.x2}$		
log Cu log y . pH _{KCl}		-0.797***
log Cu log y . log Cd		-0.558***
log Cu log y . log Pb		-0.501***
log Cu log y . log Zn		-0.461***
log Pb log y . log Cu		0.001
log Cd log y . log Cu		0.120
log Zn log y . log Cu		0.241*
pH _{KCl} log y . log Cu		0.248*
Stepwise regression		
Step no.	Variables in equation	Multiple correlation
1		log Cu 0.784
2	pH _{KCl}	log Cu 0.799
3	log Zn pH _{KCl}	log Cu 0.803
4	log Cd log Zn pH _{KCl}	log Cu 0.805
5	log Pb log Cd log Zn pH _{KCl}	log Cu 0.805

Basis of calculation: gram dry matter ($n=80$). Level of significance: *** $P<0.001$, * $P<0.05$.

which grow in closed stands in 80 sites distributed in a stratified random way in all directions within 7 km of the mill. At each site three samples were bored to a depth of 40 mm using a steel cylinder with a sharp edge (volume 154 cm³). The samples were transferred to plastic containers of a slightly larger volume, thus minimising the risk of disturbing the stratification. The material surrounding the cylinder was collected for chemical determinations. Aerobic incubation of the cylinder samples was carried out in the containers for 10 weeks (humid chamber, 22±0.5 °C, water content 47-50% of the water-holding capacity). The three samples from each site were combined for analysis. Mineral nitrogen was extracted in 1 M KCl for 2 h and an aliquot of the filtrate subjected to a two-step distillation⁸. Ammonia was determined spectrophotometrically by the indophenol method⁹. As the nitrite and nitrate fraction was small in most samples, only the increase during incubation in the sum of ammonium, nitrite and nitrate is reported. Concentrations of total Cu, Zn, Cd and Pb were determined by atomic absorption spectrophotometry after wet digestion of organic matter with HNO₃ and HClO₄. pH_{0.2 M KCl} was determined electrometrically. The data were subjected to correlation and regression analysis to quantify the relation-

Fig. 1 Relationship between copper concentration and nitrogen mineralisation rate (increase of $\Sigma\text{NH}_4+\text{NO}_2+\text{NO}_3$ during aerobic incubation of mor samples in undisturbed stratification for 10 weeks at 47-50% of the water-holding capacity and 22 °C). Double logarithmic scale.



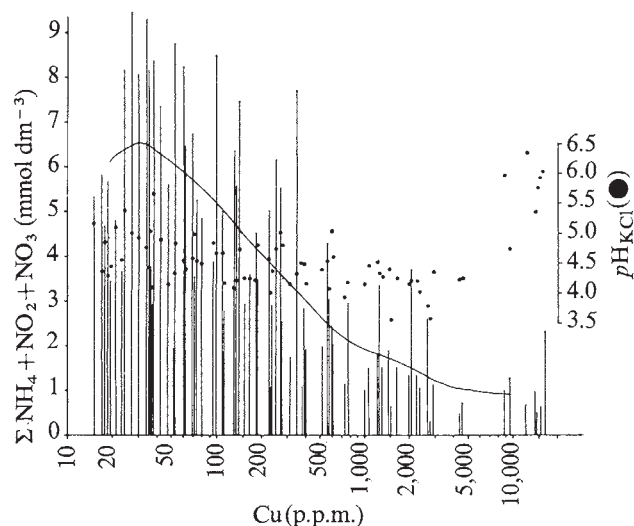


Fig. 2 As Fig. 1 but semilogarithmic scale. Increase in mineral nitrogen indicated by columns and a curve of successive means. ●, pH_{KCl} .

ship between heavy metal concentrations and biological variables. A multiple regression program was also tested (BMD 02R, step-wise regression, Health Sciences Computing Facility, UCLA, California).

The increase in mineral nitrogen was calculated as $mmol\ dm^{-3}$ considered the ecologically most appropriate basis of calculation in this case. An approximately straight regression line was obtained for increase in mineral nitrogen with Cu concentration (double log scale, Fig. 1). The partial correlation coefficients are highly significant ($P < 0.001$) for Cu but not for Zn, Cd or Pb (Table 1). The multiple correlation coefficient is somewhat improved, when pH_{KCl} is included in the equation together with Cu. Inclusion of other variables improves the coefficient very little.

More detailed information on the relationship is obtained from a semilogarithmic presentation (Fig. 2). With increasing degree of metal pollution the amount of nitrogen mineralised is reduced from 5–6 to about $1\ mmol\ dm^{-3}$. There are indications of a downward-sloping curve from a Cu concentration of about 50 p.p.m. (corresponding to three times the background level). There is some evidence for a rising curve in the interval 15–30 p.p.m., but this needs to be confirmed. The higher pH_{KCl} in heavily polluted samples is caused by the neutralising effect of the deposited metal dust, possibly also to a lower biological activity.

We conclude that heavy metal pollution, at least of Cu, is a limiting factor for the nitrogen mineralisation rate in acid forest soil. Moderate amounts of pollutants are sufficient to reduce the supply of nitrogen available to plants.

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¹ Rühling, Å., and Tyler, G., *Oikos*, **24**, 402–416 (1973).

² Tyler, G., *Pl. Soil*, **41**, 303–311 (1974).

³ Quraishi, M. S., and Cornfield, A. H., *Pl. Soil*, **35**, 51–55 (1971).

⁴ Quraishi, M. S. I., and Cornfield, A. H., *Environ. Pollut.*, **4**, 159–163 (1973).

⁵ Sandhu, M. S., and Moraghan, J. T., *Commun. Soil Sci. Pl. Anal.*, **3**, 439–447 (1972).

⁶ Tyler, G., Mörsjö, B., and Nilsson, B., *Pl. Soil*, **40**, 237–242 (1974).

⁷ Baroux, J., *C. r. heb. Séanc. Acad. Sci. Paris*, **D275**, 499–502 (1972).

⁸ Hoffmann, G., and Teicher, K., *Z. Pfl. Ernähr., Düng. Bodenk.*, **95**, 55–63 (1962).

Genetical and environmental diversity in *Drosophila melanogaster*

NATURAL populations often show extensive genetical diversity for soluble proteins¹. It is considered by some that much of

this variation has no adaptive value². In contrast, others consider that natural selection is responsible for shaping the gene pools of populations^{3–4}. Evidence exists for a genetical response to selection through environmental heterogeneity; for example, a positive and almost linear relationship was observed between the extent of the environmental heterogeneity to which laboratory populations of *Drosophila willistonii* were subjected and the average heterozygosity of an individual⁵. More recent work with *D. pseudoobscura* has been interpreted as agreeing with the latter observation⁶. These results are of interest, as in theory, genetical polymorphism can be maintained within populations living in variable environments, although the conditions for a stable equilibrium are restricted⁷.

We have tested for and assessed the proportion of loci affected by selection as a result of environmental difference, and compared the effect of uniform and variable environments on average genic heterozygosity. Detailed results and analyses will be published elsewhere. A laboratory population of *D. melanogaster* 'Texas' was used and details of the origin and maintenance of this population have already been described⁸. Genetical variation was observed in Texas at eight out of a total of 22 protein-enzyme loci studied using starch and polyacrylamide gel electrophoresis. The *pink* locus (an eye-colour mutant) was also polymorphic. The average genic heterozygosity per individual in Texas was 8.2%. On average the population size was 3,000 adults.

Six subpopulations of Texas were independently established within 3 weeks. Two replicate subpopulations, both containing 2,000 adults (equal numbers of each sex), were placed into each of the following three environments: (a) the temperature alternated between 18 and 25 °C every 3 d and food medium consisted of oatmeal, agar, molasses, live baker's yeast and sultanas; (b) kept at –15 °C for 30 min every 3 d, otherwise at 25 °C with food medium the same as for (a); (c) kept at 25 °C and food medium consisted of agar, sucrose and dried baker's yeast only. Thus, environments (a) and (b) contain more environmental variables than (c).

Our test of selection was based on genotype frequencies and preliminary investigations showed that two of the nine polymorphic loci in Texas (*Lap-A* and *Pgm*), were not suitable for this study. Activity levels of *Lap-A* were low and it was difficult to score the genotypes from a zymogram. The allele frequencies at the *Pgm* locus were 0.02 and 0.98; for reasons of labour the *Pgm* locus was not studied. Genotypes at the *pink* locus were ascertained from the progeny of backcrosses of flies to a strain homozygous for the *pink* allele.

Egg samples were taken from all of the experimental populations after 27 weeks (occasion 1), and raised to adults in bottles. The populations were resampled when they were 54 weeks old (occasion 2). The genotype frequencies were determined for all loci on 200 individuals. The *Lap-D* locus was only studied on occasion 2. As the generation time for a large population of *D. melanogaster*, kept at 25 °C and with overlapping generations, is approximately 3 weeks⁹, the populations were sampled at generations 9 and 18.

Three of the 39 χ^2 values which compare genotype frequencies in replicate populations were statistically significant. In this experiment the following items will contribute to variation between the replicate cages: variation between the samples of eggs used to form the replicate populations; variation in the samples of eggs taken from the populations at the two sampling times; random genetic drift; and when the type of selection differs in two apparently similar replicate cages, for example, the effect of environmental variation which occurs in a temporally different sequence in replicate populations¹⁰.

To determine whether the variation in genotype frequencies was greater between environments than between replicate cages, the following test was carried out: Since by definition a sum of squares (SS) = $\chi^2\sigma^2$ (where σ^2 is a theoretical variance) it follows that an observed variance $V_A = \chi^2_A\sigma^2_A/d.f.A$, where d.f.A. are the degrees of freedom of the variance A. For two observed variances V_A and V_B , V_A/V_B is a variance ratio

Table 1 Variance ratio test for environmental selection

Locus	χ^2_A Between environments		χ^2_B Replicate cages		Variance ratio for 4 and 6 d.f.	
	Occasion 1	Occasion 2	Occasion 1	Occasion 2	Occasion 1	Occasion 2
<i>Est-6</i>	34.38	13.11	3.65	4.19	14.13†	4.69*
<i>Odh</i>	9.65	11.30	4.73	17.48	3.06‡	0.97‡
<i>Pink</i>	7.92	30.21	3.23	4.35	3.68‡	10.42†
<i>Adh</i>	7.24	22.41	6.95	2.06	1.56‡	16.32†
<i>α Gpdh</i>	21.18	4.26	7.38	4.50	4.30‡	1.42‡
<i>6-Pgdh</i>	18.59	29.87	3.89	9.39	7.17*	4.77*
<i>Lap-D</i>	—	19.09	—	2.59	—	11.06†

*0.05 > P > 0.01.

†0.01 > P > 0.001.

‡Not significant.

(for which $\sigma^2_A = \sigma^2_B$ is the null hypothesis), and is equal to $(\chi^2_A/\text{d.f. A})/(\chi^2_B/\text{d.f. B})$. The consistency of the replicate cages for each locus, sampled on the two occasions, for each of the three environments was tested by a contingency χ^2 for 2 d.f. χ^2_B for 6 d.f. was obtained by summing these χ^2_2 over the three environments. For both sampling times, the genotype frequencies from replicate cages in the same environment were pooled, and χ^2_A for d.f. calculated as a test of the similarity of the genotypic distributions in the three environments.

Where there are no significant differences between replicate cages, χ^2_A is a valid and a more powerful test of selection, but if replicate cages do differ in genotype frequency then we must use the variance ratio test; we have no test for the fourth type of selection described above. Although the χ^2_A values are shown in Table 1, we have discussed our results using the variance ratio as a test for selection. This will tend to underestimate the importance of selection when there are no significant differences between replicate cages. The variance ratios are shown in Table 1, and it is seen that by occasion 2, the environments have differentially affected the genotype frequencies at all loci except *Odh* and *α Gpdh*.

This is only one measure of selection. Selection may be difficult to detect if the genotype frequencies show a similar or unidirectional responses in the three environments. As the population sizes were relatively large compared with the number of generations which lapsed between occasions 1 and 2, any effect of random genetic drift will be negligible. A difference between the genotypic distributions at the two times of sampling

would be additional evidence of selection. Such a test has been carried out by the variance ratio test described earlier, and the details are shown in Table 2. There is now evidence for selection at the *Odh* and *α Gpdh*; selection in one form or another has resulted in changes of genotype frequency at all seven loci.

The percentage heterozygosity at all loci on occasion 2 (Table 3) was transformed into angles. Analysis of variance of the transformed data showed that the variation between replicate cages was not greater than the theoretical variance σ^2 , calculated as 820.7/200. The three environments did not differ in their average level of heterozygosity (calculated over the seven loci) but the individual loci responded dissimilarly to the three environments. McDonald and Ayala's⁶ results, although not as striking as those of Powell⁷, showed an increase in the average genic heterozygosity of individuals from environments that varied in one or two levels of heterogeneity. In contrast, our experiments have shown that individuals from populations maintained in the most variable environments were not, on average, the most heterozygous. There is no *a priori* guarantee in experiments of this type, of the way in which the genetical variation will respond to the applied environmental variation. It may well be that our variable environments did not contain sufficiently effective environmental heterogeneity to bring about such results as were obtained by Powell.

We have shown, however, that the environments were sufficient to change the genotype frequencies at all of the observed loci. Analysis of variance of McDonald and Ayala's

Table 2 Variance ratio test for selection between the two sampling times

Locus	Environment	χ^2_2 Between occasions	χ^2_4 Replicate cages	Variance ratio for 2 and 4 d.f.
<i>Est-6</i>	a	5.11	3.56	2.87§
	b	0.91	1.12	1.63§
	c	48.45	3.76	25.77†
<i>Odh</i>	a	5.45	14.60	0.75§
	b	14.80	2.06	14.37*
	c	2.75	5.55	0.99§
<i>Pink</i>	a	3.09	3.06	2.02§
	b	1.03	3.50	0.59§
	c	1.13	2.01	1.12§
<i>Adh</i>	a	10.46	2.26	9.26*
	b	6.06	5.78	2.09§
	c	2.95	1.06	5.57§
<i>α Gpdh</i>	a	28.27	1.92	29.45†
	b	3.49	0.72	9.70*
	c	14.91	9.25	3.22§
<i>6-Pgdh</i>	a	9.50	7.50	2.53§
	b	8.62	1.60	10.78*
	c	27.29	1.54	35.45†

*0.05 > P > 0.01

†0.01 > P > 0.001

‡P < 0.001

§Not significant

Table 3 Percentage heterozygosity

Environment	Replicate	<i>Est-6</i>	<i>Odh</i>	<i>Pink</i>	<i>Adh</i>	α <i>Gpdh</i>	<i>6-Pgdh</i>	<i>Lap-D</i>	Average percentage heterozygosity per individual
a	1	32.9	29.8	25.8	15.6	23.6	26.3	34.8	7.55
	2	38.0	16.7	22.5	18.7	20.5	35.7	35.3	7.49
b	1	41.0	20.5	20.9	25.2	23.1	32.0	33.2	7.84
	2	43.4	15.5	27.0	23.9	24.7	29.2	35.2	7.95
c	1	45.2	20.0	16.3	19.7	30.1	20.2	25.4	7.07
	2	46.5	23.6	17.0	16.6	27.8	15.3	21.3	6.93

data after angular transformation showed, as we have done, a strong interaction between loci and the level of environmental heterogeneity. Such an interaction is again evidence for selection. The variation between environments in percentage heterozygosity was also calculated for individual loci from McDonald and Ayala's data. A χ^2 test for selection was calculated as SS/σ^2 , and eight out of the twenty tests were statistically significant.

Nevertheless, these results do not exclude the possibility that selection is acting on closely linked loci, rather than the observed loci themselves. We have tried to minimise the effect of linkage. The Texas population has been maintained in the laboratory for over 200 generations. Any non-random association between alleles caused by any initial 'founder effect' will be confined to extremely closely linked loci. Also each of the subpopulations was started with 2,000 flies. Chromosomal inversions represent extreme cases of linkage. Associations between enzyme polymorphisms and chromosomal inversions have been described in natural populations of *D. pseudoobscura*¹¹ and *D. melanogaster*^{12,13}. In *D. willistonii*, however, similar associations were not detected¹⁴. In the Texas population all of the possible combinations of alleles from linked loci can be extracted, and limited evidence has shown no evidence of inversions relevant to our study. All of the loci studied have shown evidence of selection. It is unlikely that every case of observed selection can be explained as being caused by selection at another closely linked locus.

In nature, environmental diversity is on a greater scale than that which we have created in the laboratory. As such, selection must account, directly or indirectly, for the behaviour of a substantial fraction of the genetical diversity within populations.

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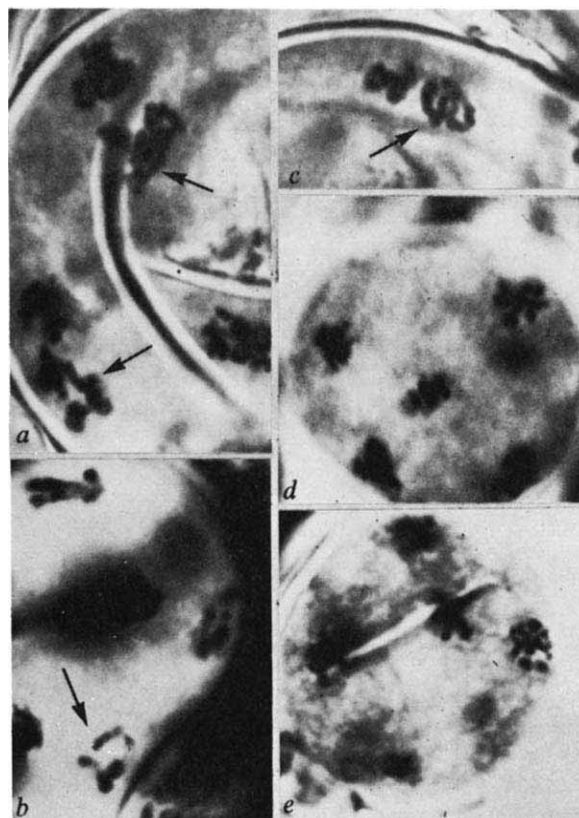
- Lewontin, R. C., *Genetical Basis of Evolutionary Change* (Columbia, University Press, New York and London, 1974).
- Kimura, M., and Ohta, T., *Nature*, **229**, 467 (1971).
- Clarke, B. C., *Nature*, **228**, 159–160 (1970).
- Richmond, R. C., *Nature*, **225**, 1025–1028 (1970).
- Powell, J. R., *Science*, **174**, 1035–1036 (1971).
- McDonald, J. F., and Ayala, F. J., *Nature*, **250**, 572–574 (1974).
- Maynard-Smith, J., *Am. Nat.*, **100**, 637–650 (1966).
- Barnes, B. W., and Kearsley, M. J., *Heredity*, **25**, 1–10 (1970).
- Barker, J. S. P., *Genet. Res. Camb.*, **3**, 388–404 (1962).
- Lewontin, R. C., in *Mathematical Challenges to the Neo-Darwinian Interpretation of Evolution*, Wistar, Symp. Monogr., **5**, 81–94.
- Prakash, S., and Lewontin, R. C., *Genetics*, **69**, 405–408 (1971).
- Mukai, T., Mettler, L. E., and Chigusa, S. I., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 1056–1069 (1971).
- Langley, C. H., Tobari, Y. N., Kojima, K., *Genetics*, **78**, 921–936 (1974).
- Powell, J. R., *Science*, **176**, 545 (1972).

palms, cocoa, citrus and rubber¹. On cocoa, where it causes black-pod disease, annual losses are conservatively estimated at 10% of the crop². Progress in disease control has been impeded by confusion over the taxonomy of the species, which has become a pigeon-hole for a number of structurally similar *Phytophthora*. This situation has been compounded by preoccupation with compatibility types, whereas morphological differences have been neglected³.

Two isolates of *P. palmivora* from cocoa in Nigeria, P131 and P132, were obtained from the Commonwealth Mycological Institute; P131 was found to be of the A2 mating or compatibility type and P132 of the A1. When mated together on carrot agar and examined cytologically, they were found to have distinctive nuclei in their sex organs. One, the large (L) chromosome type had a basic number of 5 or 6 comparatively large chromosomes at metaphase, with a multiple association of at least four chromosomes (Fig. 1a–c). The other, the small (S) chromosome type had a basic number of 9–12 much smaller chromosomes (Fig. 1d and e). On pairing P132 with an A2 isolate of *P. drechsleri* P6503 (ref. 4) it was confirmed that P132 was the L chromosome type and therefore P131 the S type.

Further cocoa isolates from Cameroun, Nigeria and

Fig. 1 a–c, Metaphase I configurations from oogonia of the L type, showing multiple associations (arrowed). d and e, Metaphase I configurations from oogonia of the S type. Aceto-orcein squash method. $\times \sim 2,200$.



Chromosome size differences in *Phytophthora palmivora*, a pathogen of cocoa

Phytophthora palmivora is one of the most ubiquitous and destructive plant pathogens of tropical tree crops such as

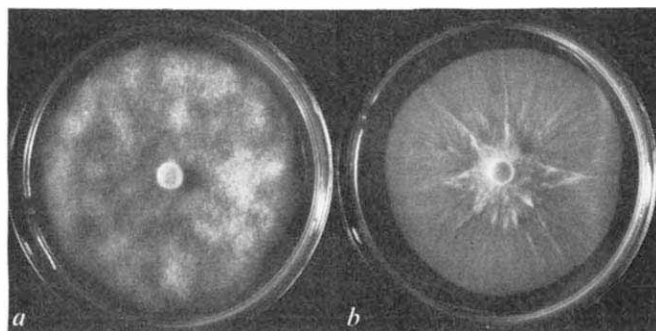


Fig. 2 Cultures of *Phytophthora* isolates from cocoa. *a*, Typical L type from Nigeria; *b*, typical S type from Ghana. Grown on carrot agar in darkness at 25 °C.

Ghana have been examined in Britain for mating type and chromosome structure. Of ten A2 isolates examined cytologically, nine were of the S and one (from Cameroun) of the L chromosome type (the latter confirmed by pairing with an A1 isolate of *P. drechsleri* P6500; ref. 4). All five A1 isolates examined were of the L type. These results establish the existence of two chromosome types of *P. palmivora* on cocoa in West Africa. They also demonstrate that both A1 and A2 compatibility types are present in the L type.

Table 2 Distribution of S and L types among *Phytophthora* isolates from cocoa in West Africa, based on chromosome structure and culture morphology

	No. of isolates*			
	L type		S type	
	A1	A2	A1	A2
Cameroun	1 (1)	1 (1)	—	2 (2)
Nigeria	430 (3)	—	—	11 (5)
Togo	—	—	—	2 (0)
Ghana	—	—	—	7 (3)

* Figures in parentheses denote number of isolates examined for chromosome structure.

(A2) is the only one associated with black-pod disease in Ghana. Behavioural differences between the two species (Table 1) may account for differences between the disease situations in Nigeria and Ghana, and should be taken into account in the application of chemical control measures and in the breeding of cocoa for disease resistance. Preliminary field observations already indicate significant pathological differences between the two species (M.J.G. and A. C. Maddison, unpublished).

The occurrence of sexually compatible L and S types on

Table 1 Comparison of large and small chromosome types of *P. palmivora*

Chromosome type	Growth rate on carrot agar (mm d ⁻¹)*	Growth in Amazon cocoa pods: lesion size (cm)†	Sporangial dimensions (µm)‡	
			Length	Length-breadth ratio
Large (L) (n = 5-6)	7.4 - 9.2	12.9 - 15.9	34.0 - 38.1	1.34 - 1.47
Small (S) (n = 9-12)	9.2 - 12.2	16.9 - 21.4	41.1 - 45.7	1.67 - 1.93

* Mean range of ten L and ten S isolates over 3 d at 25 °C in darkness.

† Lesion size, length plus breadth; mean range of nine L and nine S isolates, five replicates per isolate, after 96 h at 23 °C in the laboratory.

‡ Sporangia from cocoa-pod lesions, mounted in distilled water. Mean range of ten L and ten S isolates, 100 measurements per isolate.

L and S types could be distinguished on carrot agar, the L types (both A1 and A2) producing plentiful woolly aerial mycelium (Fig. 2a) and the S types growing faster (Table 1) and producing much less aerial mycelium with a distinctive stellate pattern in the centre of the colony (Fig. 2b). When inoculated into cocoa pods in the laboratory, L types formed a diffuse-edged surface lesion, and S types a faster developing sharp-edged lesion (Table 1). On pods the L type also formed shorter and more rounded sporangia than the S type (Table 1).

These comparative studies show that the S and L chromosome types correspond to the cocoa and rubber groups of *P. palmivora* described by Ashby⁵, the typical and atypical groups of Tucker⁶, the 'G' and 'N' forms of Turner⁷, and morphological forms 1 and 2 of Waterhouse³ respectively. The difference in chromosome size and number between the S and L types show that they must now be regarded as distinct species (details will be published elsewhere).

The occurrence of two species of *Phytophthora* on cocoa has considerable implications for the control of black-pod disease. A preliminary survey carried out to establish the distribution of the L and S types in West Africa has shown that the L type (A1) is the principal disease-causing organism in Nigeria (Table 2), the S type (A2), with one exception, having been isolated only from soil or rotting husks beneath cocoa trees. In contrast the survey results, together with Turner's morphological studies⁷, indicate that the S type

cocoa in Nigeria and elsewhere raises the possibility of hybridisation between them, and of the occurrence of intermediates. So far hybrids have not been identified. It is likely that they would have reduced viability and that only selfed oospores would survive in nature.

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- 1 Chee, K. H., *Rev. Applied Mycol.*, **48**, 337-344 (1969).
- 2 Padwick, G. W., *Phytopath. Pap. No. 1*, 1-60 (Commonwealth Mycological Institute, 1956).
- 3 Waterhouse, G. M., in *Phytophthora Disease of Cocoa* (edit. by Gregory, P. H.), 28-36 (Longmans, London, 1974).
- 4 Brasier, C. M., and Sansome, E., *Trans. Br. mycol. Soc.* (in the press).
- 5 Ashby, S. F., *Trans. Br. mycol. Soc.*, **14**, 18-38 (1929).
- 6 Tucker, C. M., *Res. Bull. Mo. agric. Exp. Sta. no. 153-173* (1931).
- 7 Turner, P. D., *Trans. Br. mycol. Soc.*, **43**, 665-672 (1960).

Direct magnetic separation of red cells from whole blood

THE magnetic properties of haemoglobin are well established¹. The prosthetic group of haemoglobin has a protoporphyrin structure (ferrohaem) in which the iron atom is ionically bound. Consequently there are in deoxyhaemoglobin four unpaired electrons per iron atom leading to a paramagnetic moment of 5.35 Bohr magnetons per haem group. Oxyhaemoglobin on the other hand is diamagnetic. Other iron-containing haemoproteins such as ferrihaemoglobin have been shown by magnetic susceptibility measurements² to possess magnetic moments per haem group which correspond reasonably closely to those predicted from the number of unpaired electrons per iron atom. Consequently red blood cells containing deoxyhaemoglobin when placed in a magnetic field B_0 (Tesla) with field gradient dB_0/dz may be expected to experience a magnetic force F_M (Newtons) in the z direction given by

$$F_M = (\chi V/\mu_0)B_0(dB_0/dz) \quad (1)$$

where χ is the (SI) susceptibility of the red blood cell, V (m^3) its volume and μ_0 the permeability of free space.

In spite of this known property the only previous recorded attempt by Levine³ to increase the sedimentation rate of red cells in whole blood by placing them in a magnetic field gradient was unsuccessful. According to our own estimates this null result is not surprising as for the low field gradient operative in Levine's experiment (approximately 0.1 T m^{-1}) the force predicted by equation (1) is only a few per cent of the gravitational force experienced by the red cells. The development of a new type of high gradient magnetic separator, as described by Oberteuffer⁴, which enables magnetic field gradients of the order of 10^4 – 10^5 T m^{-1} to be achieved has led us to reassess the possibility of the magnetic separation of blood components. We believe that we have demonstrated that HGMS can be used as an effective means of separating red cells from whole blood.

In the present experiment a polyethylene cylinder of 60 ml capacity with inlet and outflow arrangement was mounted between the pole-pieces of a conventional electromagnet. Into this cylinder smooth stainless steel wire of circular cross section and 25 μm diameter was randomly packed to a volume of 40 ml. Approximately 2% by volume of this 40 ml 'filter' was actually occupied by wire. The length of the filter was less than the diameter of the magnet pole pieces ensuring that the whole wire volume was exposed to a uniform magnetic field. A standard magnetic field of 1.75 T (17.5 kgauss) was used and the magnetic field gradient close to the wires was estimated to be $8 \times 10^3 \text{ T m}^{-1}$. Isotonic sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) was used both as a reducing agent and as a carrier for the whole blood.

The sample used was whole human blood with ACD anticoagulant in which most of the plasma had been replaced by $\text{Na}_2\text{S}_2\text{O}_4$ solution. The haemoglobin concentration of this standard sample was 11.8 g dl^{-1} . The column was first flushed out with $\text{Na}_2\text{S}_2\text{O}_4$ solution and then with the magnetic field of 1.75 T switched on, 5 ml of the blood sample was applied to the top of the column. This was followed by 270 ml of $\text{Na}_2\text{S}_2\text{O}_4$ solution, introduced slowly to maintain the flow rate through the column at approximately 4.5 ml min^{-1} . From the point when the blood was introduced samples flowing from the column were taken as a function of time. After a certain amount of strong discoloration with red cells or haemoglobin the effluent developed an almost transparent pale pink colour. After approximately 175 ml of solution had passed through the column the magnetic field was reduced to zero and successive 5 ml aliquots were taken. The effluent was observed to change markedly to a deep red colour as the field was

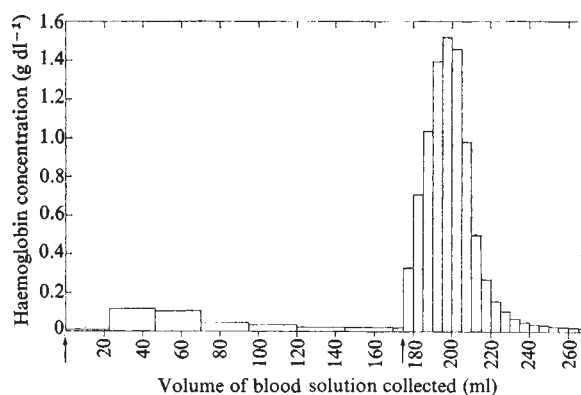


Fig. 1 Haemoglobin concentration of samples as a function of volume flowing through the magnetic separator. First arrow, field on, blood applied; second arrow, field off.

switched off. All aliquots were spun to check for cell lysis and then analysed for haemoglobin content.

Figure 1 shows the measured haemoglobin concentration of the effluent as a function of time throughout the whole experiment. It can be seen that approximately 70% of the red cells applied to the column were retained until the field was removed. (This implies that 1.30×10^{10} red blood cells were held in the filter.)

Studies of the aliquots showed no evidence in the form of free haemoglobin for immediate red cell destruction. This test was of particular interest as preliminary experiments with 'household' steel wool as the filter medium, although producing similar separation effects, led to a large number of ruptured or severely damaged red cells. The probable reason is that this material possesses an extremely irregular and non-uniform surface.

Scanning electron microscope studies of the cells filtered using the regular stainless steel wire indicated that a small proportion possessed slight morphological abnormalities, mostly in the form of small surface protruberances or indentations. We have yet to determine whether such defects affect the ultimate viability of the red blood cells.

An estimate of the surface area of the filter medium indicates that the wire was completely covered with red cells during the time that the magnetic field was applied. This conclusion is supported by the constant 'hold-back' value of 0.06 g haemoglobin per g wire observed in a number of experiments.

In magnetic separation terms the flow velocity used in the present experiment is extremely low ($\sim 10^{-4} \text{ m s}^{-1}$) and further work is necessary to determine how the filter performance varies with flow rate and applied magnetic field.

The successful outcome of this preliminary experiment indicates that high gradient magnetic separators may be used to produce either blood plasma with a low red cell population, or a suspension of red cells free from other blood contents. It also suggests that other haemoproteins in suitable form may be separated in the same way. The application of this principle to specific research or clinical applications is of interest for further investigation.

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¹ Taylor, D. S., and Coryell, C. A., *J. Am. Chem. Soc.*, **60**, 1177–1181 (1938).

² Coryell, C. A., Stitt, F., and Pauling, L., *J. Am. Chem. Soc.*, **59**, 633–42 (1937).

³ Levine, S., *Science*, **123**, 185–186 (1956).

⁴ Oberteuffer, J. A., *IEEE Trans. Magn.*, **MAG-9**, 303–306 (1973).

Study of cell development using derepressed mutations

CURRENTLY there is considerable interest in determining how genes are expressed in a highly ordered sequence during morphogenesis. The main genetic approach to this problem has been to study mutants blocked in development. Such mutants are, however, highly pleiotropic; no biochemical or morphological event normally occurring after a point of mutational block is expressed¹. This, coupled with inadequate knowledge of biochemical events specific to development², and the absence of detectable cross feeding between defective mutants³, makes it difficult to determine the precise lesion in any mutant and its place in control of the sequence. Another method, selecting mutations affecting known biochemical functions can be more rewarding but is also hampered by ignorance of biochemical events relevant to development^{2,4}.

In these circumstances a useful beginning to genetic study of developmental control is to isolate mutants which undergo development in conditions in which the wild type does not. Such derepressed mutants will be altered in the initiation of morphogenesis. As we are dealing here with microbial sporulation, the term initiation is used for the complex of metabolic and genetic events necessary to set sporulation in train.

Derepressed mutants are important because they are probably defective in control functions, and indicate the number and nature of elements involved in initiation. Moreover, they provide strains from which to select further mutations affecting initiation and the first irreversible steps of the sporulation sequence. By genetic analysis of the interaction between these mutations (for example tests of dominance, position effects and mapping) it is possible to make sensible statements about control. Progress in the genetics of later control elements in the morphogenetic sequence may also be made by judicious mutant selection.

Yeast sporulation is a suitable system in which to select such mutants. Yeast is a simple eukaryote amenable to genetic study and culture conditions leading to initiation of sporulation are known. As in many other microorganisms these are similar to those leading to relief of catabolite repression by both carbon and nitrogen sources⁵⁻⁷.

Completely derepressed sporulation mutants must be conditional, as once initiated to sporulation a cell is incapable of contributing to population growth⁸. To date two mutants (ID16D and ID19D) which do not sporulate during vegetative growth on YEPD medium (1% yeast extract, 2% peptone, 2% glucose), but unlike the wild type form ascospores at high frequency during starvation in this medium, have been isolated from the homothallic diploid *Saccharomyces cerevisiae* strain S41 (Fig. 1). Mutation to this derepressed phenotype is a rare event occurring at a frequency of less than 1 in 10⁸ survivors after ultraviolet mutagenesis. Study of conditions affecting sporulation in both wild type and mutant strains showed that both mutants had lost nitrogen-source repression, but were still subject to glucose repression. In a minimal medium containing glucose and (NH₄)₂SO₄, both mutants sporulated in the presence of excess NH₄⁺, but sporulation was repressed by excess glucose (Table 1).

Mutants no longer subject to nitrogen repression should sporulate extensively on media in which glucose is replaced by poorer, non-fermentable carbon sources. This was indeed the case for both mutants; on media containing glycerol sporulation was so rapid and extensive that they grew poorly whereas the wild type strain grew normally and sporulated poorly (Fig. 2).

It is unlikely that this effect is caused by a mutation

Table 1 Sporulation of strains 1D16D and S41 in minimal media

Culture medium* Glucose conc. (mg ml ⁻¹)	(NH ₄) ₂ SO ₄ conc. (mg ml ⁻¹)	Strain	Sporulation† (% asci)	Turbidity† (600 nm)
0.5	10	1D16D	30	0.75
		S41	0	0.76
1	10	1D16D	30	1.5
		S41	0	1.4
5	10	1D16D	10	4.6
		S41	0	3.7
10	10	1D16D	0	5.1
		S41	0	3.8
10	0.05	1D16D	0.5	1.2
		S41	0.1	1.1
10	0.1	1D16D	0	2.5
		S41	0	2.5
10	0.5	1D16D	0	5.1
		S41	0	3.8
10	1	1D16D	0	5.2
		S41	0	3.7
Galactose	MIN‡	1D16D	15	0.4
		S41	0	3.7

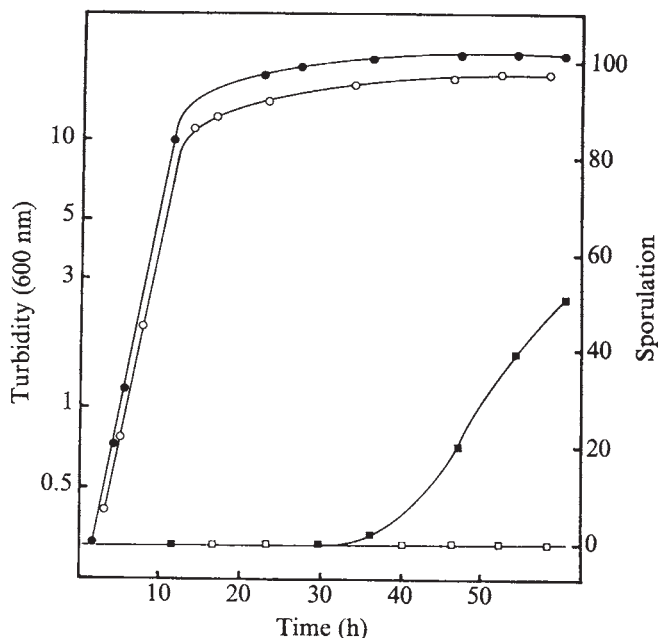
*Cultures were pregrown in YEPD, washed in minimal salts and inoculated at turbidity 0.1 into minimal medium containing: Wickerham minimal salts; arginine at 25 mg ml⁻¹ and stated concentrations of glucose and (NH₄)₂SO₄.

†% Sporulation and turbidity were estimated after 5 d incubation at 30°C.

‡Galactose minimal medium contained salts and arginine as before, galactose at 20 mg ml⁻¹ and (NH₄)₂SO₄ at 1 mg ml⁻¹.

affecting solely glycerol metabolism as similar results have been obtained on replacing glucose with other carbon sources (including galactose and acetate) causing less repression. This mutation cannot be dismissed simply as causing a

Fig. 1 Growth and sporulation of wild type and derepressed strain 1D16D on YEPD medium. Wild type strain S41 a/a D/D arg 4-1/arg 4-1 cyh-1 cyh-1 spores were mutagenised according to standard methods¹⁰. Spores were germinated in YEPD and incubated for approximately 1 week at 34°C. Cultures were treated with ether as described previously¹¹ and resuspended in YEPD to allow germination of viable spores. This extended incubation followed by ether treatment was repeated and derepressed mutants were recovered by plating survivors of the final ether treatment on YEPD. S41 is homothallic and allows recovery of recessive mutations expressed only in diploids¹⁰. Growth was determined turbidometrically after inoculating cultures to turbidity 0.1 from an exponential YEPD culture. Sporulation was estimated by direct counting of percentage asci. ○, Growth of S41; ●, growth 1D16D; □, sporulation of S41; ■, sporulation of 1D16D.



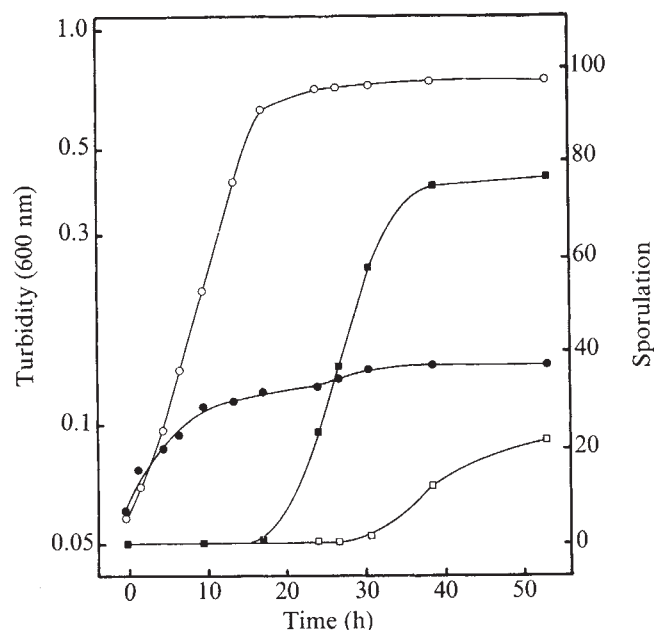


Fig. 2 Growth and sporulation of wild-type and derepressed strain ID16D on glycerol-containing medium. Growth and sporulation (see legend Fig. 1) were estimated on cultures inoculated from YEPD into a fivefold dilution of YEPG medium (1% yeast extract, 2% peptone, 3% v/v glycerol). ○, Growth of S41; ●, growth of ID16D; □, sporulation of S41; ■, sporulation of ID16D.

defect in general cellular metabolism and irrelevant to initiation of sporulation. First, sporulation is not an automatic response to the cessation of growth; the conditions leading to normal sporulation are quite specific and auxotrophic mutants do not sporulate in the absence of the requirement⁶. Secondly, the mutation does lead to sporulation in conditions where the wild type does not sporulate and the effect must therefore be associated in some way with initiation. It may be that this connection is relatively indirect, although reversion results discussed later indicate a fairly direct effect of the lesion. Finally, regardless of the nature of the primary lesion, mutants of this type are important as they are valuable tools for selecting second-site mutations affecting sporulation initiation. This is very useful as mutation to derepression is a fairly rare event.

It is clear that nitrogen-source repression of yeast sporulation can be separated from that due to a carbon source, and there seem therefore to be at least two distinct systems (other than mating-locus control) repressing sporulation. It is also likely that completely derepressed sporulation will be the result of at least two mutations. The mutants described here are ideal for trying to isolate the completely derepressed phenotype and also to test the hypothesis that sporulation in yeast is repressed by the same mechanism(s) as synthesis of inducible enzymes in carbon and nitrogen-source repression.

Genetic study has shown that for each mutant nitrogen derepressed sporulation is the result of a single, nuclear mutation. The mutations are recessive to wild type in both sporulation and ability to grow on YEPG. They fail to complement each other, although recombination between the two has been detected at low frequency. The mutations therefore probably represent alleles of the same gene and are designated *spd* 1-1 and *spd* 1-2 for strains ID16D and ID19D, respectively. The *spd* 1 mutation is not linked to the mating-type locus.

Strain ID16D grows so poorly on YEPG that revertants can be isolated easily; the diploid strain reverts spontaneously, or after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The majority of revertants tested (69 out of 80) were also revertant to wild type or pseudo wild type

sporulation (that is, sporulate on potassium acetate media, but not after starvation on YEPD). Of the other growth revertants, five were asporogenous and six were particularly interesting in that they showed wild type repression at 20 °C, but were asporogenous at 34 °C.

The reversion results are important. First, the coreversion of the two characters, derepressed sporulation and inability to grow on non-repressing carbon sources, indicates that these are interrelated effects of the same mutation. This has been confirmed by meiotic analysis. Secondly, by selecting for revertants which can grow in conditions favouring derepressed sporulation (that is, on YEPG) we have found that in most, if not all, cases the reversion event altered only the ability of the strain to be initiated to sporulate, and not its capacity to complete spore formation in conditions of complete derepression (on potassium acetate). This is a special case confirming our previous prediction that only those organisms defective in initiation of sporulation will have selective advantage for growth under conditions favouring sporulation rather than growth⁹. This means that reversion of *spd* strains, and competitive growth selection of sporulation-defective mutants from the wild type, are two methods of rapidly obtaining a wide range of mutations in all elements of the initiation/repression mechanism.

The revertants for growth on glycerol which were simultaneously temperature-sensitive for sporulation are particularly interesting as these may be defective in a function (or functions) specific to the initiation of sporulation.

In summary, mutants defective in nitrogen-source repression of yeast sporulation have been isolated and used to study mechanisms initiating sporulation. Sporulation is repressed by at least two separate systems, one caused by the carbon substrate, the other by the nitrogen source. Nitrogen derepressed mutants (*spd* 1) are unable to grow on media containing a poor carbon source and are useful strains in which to select further mutations affecting initiation, and eventually, the first irreversible step(s) of sporulation. These results are the first in an approach to studying genetic regulation of development by selecting mutants undergoing morphogenesis when the wild type does not; in other words, beginning at the beginning.

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- 1 Waites, W. M., et al., *Biochem. J.*, **118**, 667-676 (1970).
- 2 Dawes, I. W., and Hansen, J. N., *Crit. Rev. Microbiol.*, **1**, 479-520 (1972).
- 3 Mandelstam, J., *Symp. Soc. gen. Microbiol.*, **19**, 377-401 (1969).
- 4 Hanson, R. S., Peterson, J. A., and Yousten, A. A., *A. Rev. Microbiol.*, **24**, 53-90 (1970).
- 5 Schaeffer, P., Millet, J. and Aubert, J. P., *Proc. natn. Acad. Sci. U.S.A.*, **54**, 704-711 (1965).
- 6 Dawes, I. W. and Mandelstam, J., *J. Bact.*, **103**, 529-535 (1970).
- 7 Miller, J. J., *Nature*, **196**, 214-215 (1963); *Can. J. Microbiol.*, **9**, 259-277 (1963).
- 8 Dawes, I. W., and Thornley, J. H. M., *J. gen. Microbiol.*, **62**, 49-66 (1970).
- 9 Dawes, I. W., thesis, Oxford Univ. (1969).
- 10 Esposito, M. S., and Esposito, R. E., *Genetics*, **61**, 79-89 (1969).
- 11 Dawes, I. W., and Hardie, I. H., *Molec. gen. Genet.*, **131**, 281-281 (1974).

Cytotoxicity of isolated plasma membranes from lymph node cells

DESTRUCTION of certain other cells on contact is a specialised capability of some cells of the lymphoid system. Our recent study has indicated that the actual lysis results from osmotic effects¹, but the biochemical process which precedes this step is unknown². If the effector cells are killed before they come into contact with the target cells, cytotoxicity is abolished². Once contact of effector cells and target cells is established, however, killing of the effector cells with specific antibody and complement does not inter-

rupt the lysis of target cells, which continues to completion^{3,4}. These observations suggest that viable lymphocytes are required to establish the correct type of contact with target cells (probably because lymphocyte motility is involved^{2,5,6}), but that once effective contact has occurred lymphocyte viability is no longer necessary. Since contact of plasma membranes of lymphocytes with those of target cells is required for the lytic process², we have examined the possibility that the plasma membranes of lymphoid cells themselves have cytolytic capacity. This interpretation would be consistent with our previous report⁷ that subcellular sedimentable fractions from livers of mice infected with *Mycobacterium tuberculosis* (BCG), and heavily infiltrated with mononuclear cells, are lytic for tumour cells.

Lymph node cells were obtained from mice 4 d after contact sensitisation with oxazolone. Such contact sensitisation provides a powerful stimulus for early blast transformation and mitosis of T-lymphocytes (maximum 3-4 d) as well as some later stimulation of B-lymphocytes (maximum 5-8 d)⁸. As shown previously⁹ and in Table 1, lymph node cells taken from mice sensitised with oxazolone are able to lyse P-815-X2 mastocytoma cells, especially in the presence of phytohaemagglutinin (PHA).

In further experiments lymph node cells were broken by shearing in controlled conditions¹⁰. Fractions considerably enriched in plasma membranes were obtained by sucrose-density-gradient centrifugation. As a marker for plasma

Table 1 Cytolytic activity of activated lymph node cells

Ratio of lymph node cells to mastocytoma cells	PHA	% Lysis of mastocytoma cells
50:1	+	61.2±1.3
50:1	-	7.9±0.4
30:1	+	40.5±0.7
30:1	-	6.3±0.2

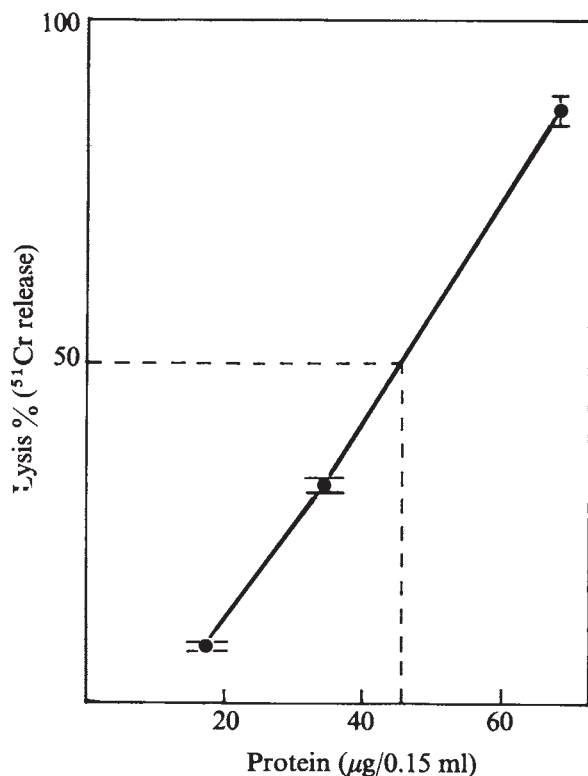
Lymph node cells were obtained from the regional lymph nodes of CBA mice which had been skin-sensitised with 4-ethoxymethylene-2-phenyl oxazolone (BDH) 4 d earlier⁹. Lymph node cells were incubated with 10^5 ^{51}Cr labelled P-815-X2 mouse mastocytoma cells in 1 ml Eagle's medium with 5% foetal bovine serum in duplicate tubes for 6 h at 37 °C in the presence or absence of 2 μg PHA (Wellcome). Lysis of mastocytoma cells was assessed by the percentage of ^{51}Cr released from the cells as a proportion of that released by freezing and thawing and with the label released in the absence of lymph node cells subtracted².

membrane 5'-nucleotidase and as a marker for lysosomal membranes β -glucuronidase were used¹¹. Lysis of P-815-X2 cells was measured by ^{51}Cr release². For each fraction a concentration-response curve was established (Fig. 1), and the relative cytolytic potencies of fractions were expressed as the reciprocal of the protein concentrations required for 50% lysis of target cells after 6 h of incubation. Purified plasma membrane fractions of lymph node cells had considerable cytolytic activity (Figs 2 and 3). In all experiments cytolytic activity was maximal in low density fractions expected to contain plasma membranes¹⁴ and was positively correlated with 5'-nucleotidase activity (Fig. 3) but negatively correlated with β -glucuronidase activity.

These results show that plasma membranes of stimulated lymph node cells have considerable cytolytic potential, much greater than exerted by lysosomal or other fractions. The cytotoxicity of purified plasma membrane preparations contrasts with the lack of such activity in whole homogenates. The purified plasma membranes, probably in the form of microvesicles, are able to diffuse freely and establish contact with the plasma membranes of target cells, whereas in homogenates or killed cells the plasma membranes are entrapped in a gelatinous mass comprising cytoplasmic and nuclear contents. An approximate indication of the efficiency of the plasma membranes in killing target cells can be obtained by comparing the 5'-nucleotidase activity in purified plasma membrane fractions required for lysis of 50% target cells in 6 hours (37-52 nmol Pi per h per $\approx 10 \mu\text{g}$ protein) with the concentration of this enzyme in the number of viable lymph node cells which have the same cytolytic activity (8-20 nmol Pi per h per $\approx 10^6$ cells) in the presence of PHA. The motility of the intact cells would increase the chances of establishing effective contact with target cells and possibly some of the membranes, being "inside out" would have diminished cytolytic activity. Nevertheless, the remarkable coincidence of cytolytic activities of whole cells and plasma membranes observed suggests that the plasma membranes may act as mediators of the cytolytic effects of lymph node cells.

Other proposed mechanisms of cell mediated cytotoxicity are lymphotoxin, and the possible action of lysosomal enzymes. Lymphotoxin is a soluble cytotoxic product of stimulated lymphocytes and macrophages^{15,16}, the reported rate of action of which is much slower than the cytotoxicity produced by our plasma membrane preparations. Our method of preparation of membranes should eliminate highly soluble lymphotoxin from the system. Evidence which indicates that lysosomal enzymes might be cytotoxic in an extracellular location, in the absence of phagocytosis, seems to be lacking¹⁷. Furthermore, we have shown the least activity of the lysosomal marker, β -glucuronidase, in fractions richest in plasma membranes and in cytolytic activity. Our data seem consistent with a system intrinsic

Fig. 1 Concentration-response curve for lysis of P-815-X2 mastocytoma cells by a plasma membrane enriched fraction from lymph node cells collected at the 39/36% interface of a sucrose density gradient (see Fig. 2). To 2×10^4 ^{51}Cr labelled mastocytoma cells (see Table 1) in 0.1 ml of Eagle's medium containing 3.5% foetal bovine serum in plastic tubes, twofold dilution of the fraction suspended in 0.05 ml saline was added and the mixtures incubated at 37 °C with occasional shaking for 6 h. Then 0.2 ml of medium was added, the cells centrifuged and 0.2 ml of supernatant taken for counting of radioactivity. The concentration of protein in a fraction required to lyse 50% of the cells was determined by interpolation. The protein content was measured by the method of Lowry using bovine serum albumin as standard. In this example 46 μg protein was required for 50% lysis, and cytolytic activity of the fractions was expressed as the reciprocal (100/46).



to plasma membranes, rather than either of these alternative mechanisms.

The cytolytic plasma membrane fractions obtained from the lymph nodes were probably in greater part from T-lymphocytes, but B-lymphocytes and other supporting tissue cells as a source have not been excluded. The lymph node cells contained fewer than 4% macrophages, and removal of histochemically detectable macrophages by adsorption on cotton wool¹⁸ did not diminish the cytolytic capacity of isolated membranes. It remains to be examined whether the cytolytic activity is confined to the membranes of

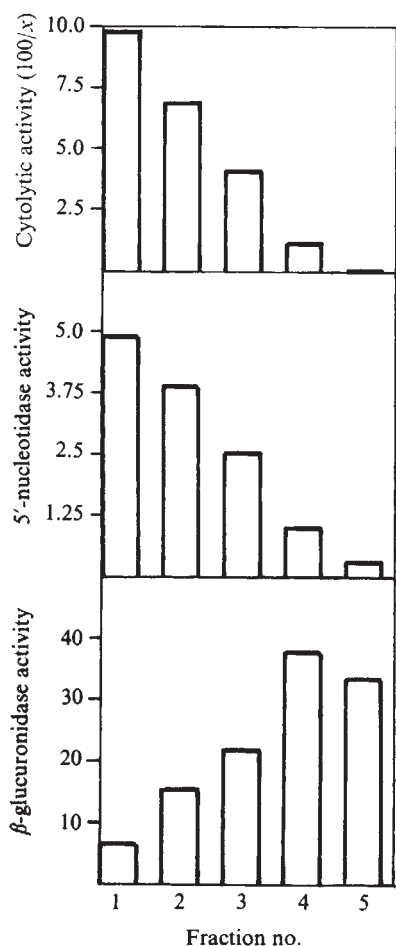


Fig. 2 Relationship between cytolytic activity, 5'-nucleotidase activity, and β-glucuronidase activity of different subcellular fractions from sensitised lymph node cells (see Table 1). One volume of packed cells (0.5–1.0 ml) was suspended in 9 volumes of saline containing 10 mM Tris-HCl buffer at pH 7.4 and 1 mM Mg Cl. The cells were disrupted by forcing them through a spring-loaded valve in a pump designed by Wright *et al.*¹⁰. One volume of the cell homogenate was mixed at 4°C in a Teflon-rotary homogeniser with two volumes of 60% w/w sucrose in 10 mM Tris at 7.4 pH. Five ml of this mixture was overlaid in a tube of 15 × 97 mm with 2 ml volumes of 39, 36, 34, 32 and 30% sucrose (w/w) solution and the tubes centrifuged in a swing-out rotor at 75,000g for 12 h. The fractions collected at these sucrose densities were diluted and sedimented at 70,000g for 1.5 h, resuspended in Tris-saline, sedimented again and finally resuspended in small volumes of Tris-saline. 100/X is the reciprocal of the protein concentration (X, in μg) of a fraction needed for 50% lysis of mastocytoma cells (see Fig. 1). 5'-nucleotidase activity is expressed as nmoles of inorganic phosphate liberated from adenosine-5'-monophosphate (Boehringer) at pH 8.5 by 1 μg protein of a fraction per hour using the method of Widnell and Unkeless¹². β-glucuronidase activity is presented as pmol of phenolphthalein released from phenolphthalein glucuronic acid per μg protein per h by the method of Talalay *et al.*¹³. The fractions are numbered as follows: 1 = < 32% sucrose, 2 = 32–34% sucrose, 3 = 36–39% sucrose, 4 = 39–43% sucrose, 5 = whole homogenate.

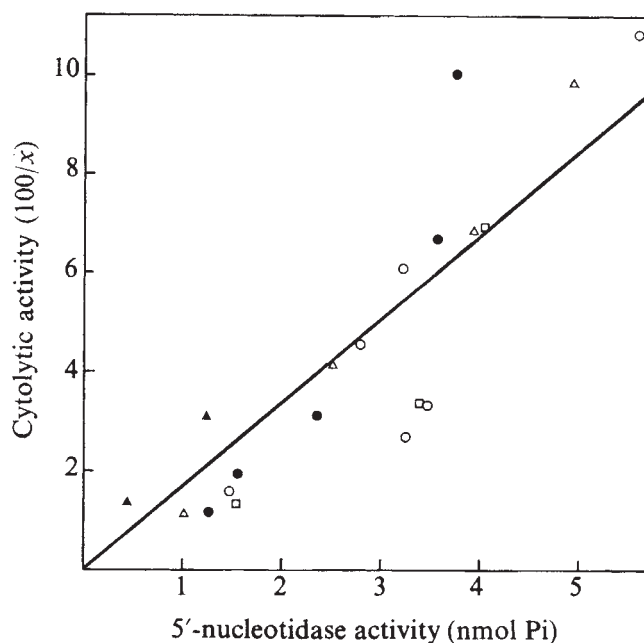


Fig. 3 The relationship of cytolytic and 5'-nucleotidase activities in several fractions from five different membrane preparations. Activities are expressed as in Fig. 2. Analysis of variance (performed by Mr M. J. R. Healey) showed that a single line through the origin provided a satisfactory fit for all the observations, with no significant differences in slope or intercept between the five sets. The slope was 0.168 ± 0.011 showing a highly significant positive correlation ($P \leq 0.001$).

stimulated lymphocytes, the nature of the cytolytic activity of these membranes, and the role that it may have in cell-mediated cytotoxicity.

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- ¹ Ferluga, J., and Allison, A. C., *Nature*, **250**, 673–675 (1974).
- ² Cerottini, J. C., and Brunner, K. T., *Adv. Immun.*, **18**, 67–132 (1974).
- ³ Martz, E., and Benacerraf, B., *J. Immun.*, **111**, 1538–1545 (1973).
- ⁴ Sanderson, C. J., and Taylor, G. A., *Cell Tissue Kinet.*, **8**, 23–32 (1975).
- ⁵ Ferluga, J., Asherson, G. L., and Becker, E. L., *Immunology*, **23**, 577–590 (1972).
- ⁶ Cerottini, J. C., and Brunner, K. T., *Nature new Biol.*, **237**, 272–273 (1972).
- ⁷ Ferluga, J., *Lancet*, **ii**, 1476–1477 (1973).
- ⁸ Davies, A. J. S., Carter, R. L., Leuchars, E., and Wallis, V., *Immunology*, **17**, 111–126 (1969).
- ⁹ Asherson, G. L., and Ferluga, J., *Immunology*, **25**, 471–483 (1973).
- ¹⁰ Wright, B. M., Edwards, A. J., and Jones, V. E., *J. Immun. Meth.*, **4**, 281–296 (1974).
- ¹¹ DePierre, J. W., and Karnovsky, M. L., *Science*, **183**, 1096–1098 (1974).
- ¹² Widnell, C. C., and Unkeless, J. C., *Proc. natn. Acad. Sci. U.S.A.*, **61**, 1050–1057 (1968).
- ¹³ Talalay, P., Fishman, W. H., and Huggins, C., *J. biol. Chem.*, **166**, 757–772 (1946).
- ¹⁴ Crumpton, M. J., and Snary, D., *Contemporary Topics in Molecular Immunology*, **3** (edit. by Ada, G. L.) (Plenum, London, 1974).
- ¹⁵ David, J. R., and David, R. R., *Progr. Allergy*, **16**, 300–449 (1972).
- ¹⁶ Kramer, S. L., and Granger, G. A., *Cell Immun.*, **15**, 57–68 (1975).
- ¹⁷ Taubman, S. B., and Cogen, R. B., *Proc. Soc. exp. Biol. Med.*, **145**, 952–957 (1974).
- ¹⁸ Hogg, N. M., and Greaves, M. F., *Immunology*, **22**, 959–965 (1972).

Cytotoxicity of alveolar macrophages for virus-infected cells

MANY studies have emphasised the importance of cell-mediated immunity in virus infections^{1–4} and this has been measured *in vitro* by leukocyte-mediated killing of virus-infected target cells^{1–3}. Although several investigators have concluded that the active cells *in vitro* are specifically sensitised T lymphocytes^{6,9}, a similar activity for peritoneal macrophages has been suggested⁵. The role of alveolar macrophages in virus infections of the respiratory tract

has, however, received little attention. Therefore, in a study of immunity to respiratory disease of cattle, the activity of cells washed from the lungs was investigated. This report describes experiments demonstrating the ability of such cells to kill parainfluenza virus-infected cells.

Lungs from freshly slaughtered calves were washed out with phosphate buffered saline (PBS) and the resulting cell suspension centrifuged at 500g for 20 min. The cell pellet was suspended at about 10^7 cells ml^{-1} in Eagle's Basal Medium containing 2% heated foetal calf serum (BMS). This preparation is subsequently referred to as lung wash cells (LWC). Lymph node cell (LNC) suspensions were prepared by teasing roughly minced bronchial lymph nodes in BMS and peripheral blood mononuclear cells (PBL) were isolated from heparinised whole blood by centrifugation on Ficoll-Triosil gradients¹⁰. The viability of all cell preparations exceeded 90%, by Trypan blue exclusion.

The cytotoxicity of these cells was estimated by measuring release of ^{51}Cr from infected calf kidney cells. The calf kidney (CK) cell suspensions were prepared by trypsinisation of primary monolayers, inoculated with at least 50 plaque forming units (PFU) of parainfluenza-3 (Pi-3) virus per cell and labelled with 40 μCi ^{51}Cr (sodium chromate, 100–300 mCi mg^{-1} Cr, Radiochemical Centre, Amersham) per 10^6 cells.

Fluorescent antibody staining of unfixed CK cells infected with Pi-3 virus showed that viral antigens were present on the cell surface in maximal amounts and in 100% of cells at 18 h. Therefore, in the cytotoxicity assay, target cells were always used at this time.

Washed, labelled CK cells (infected or uninfected) were incubated with the LWC, LNC or PBL preparations in scintillation vials for 18 h at 37 °C. After centrifugation

(300g for 10 min) the radioactivity in cells and supernatant fluids was measured separately in a gamma counter and specific ^{51}Cr release calculated (Tables 1 and 2). Incubation of cell mixtures for longer than 18 h did not result in a significant increase in specific ^{51}Cr release.

Results of preliminary experiments are shown in Table 1. Significant specific ^{51}Cr release from Pi-3 virus-infected target cells was detected with LWC but not with LNC or PBL. Specific ^{51}Cr release from uninfected target cells never occurred. Cytotoxicity was shown by LWC from one gnotobiotic and four conventional calves which had recovered from Pi-3 virus infection. Activity was also shown by LWC from a control gnotobiotic calf, never exposed to Pi-3 virus. Further experiments (Table 2, Fig. 1) used LWC from 6 and 9-d-old normal calves and these cells showed comparable cytotoxic activity.

The spontaneous ^{51}Cr leakage from virus-infected target cells was about 30% (Table 1). By infecting and labelling target cells simultaneously as described in Table 2 leakage was reduced to about 22%, thus increasing the sensitivity of the assay. By this method specific ^{51}Cr release by LWC from calf 5 was increased from 21% (Table 1) to 38%.

Hyperimmune antiserum inhibited LWC cytotoxicity (Table 1), and this effect was examined further. At dilutions of 10^{-1} to 10^{-3} the antiserum inhibited cytotoxicity by more than 80% and inhibition was still detectable at a dilution of 10^{-4} (Table 2).

Six different ratios of LWC to target cells were investigated (Fig. 1). There was an approximate linear increase in the amount of specific ^{51}Cr release as the ratio was increased from 5:1 to 50:1. Thereafter no further increase in ^{51}Cr release was measured up to a ratio of 200:1.

These experiments demonstrate that bovine LWC have

Table 1 Cytotoxicity of various calf cell populations for Pi-3 virus-infected CK cells

Calf no.	Age (months)	Previous Pi-3 virus infection	Cell type	Antiserum*	% Specific ⁵¹ Cr release† from Pi-3 virus-infected cells		
Conventional	1	7.5	+	LWC	—	26.8	
				LNC	—	0.4	
	2	7.5	+	LWC	—	26.5	
				LNC	—	4.0	
	3	7	+	LWC	—	25.6	
				LNC	—	0.8	
	4	8	+	LWC	—	23.9	
				LNC	—	1.1	
Gnotobiotic	5	2.5	+	LWC	—	20.5	
				LNC	—	1.6	
				PBL	—	0	
				LWC	+	5.2	
	6	1.5	—		LNC	+	2.1
					LWC	—	16.2
					LNC	—	0
					PBL	—	0
				LWC	+	4.6	
				LNC	+	0.5	
Medium Controls			None	—	0		
			None	+	0		

Suspension cultures of 10^7 virus-infected or uninfected secondary CK cells in 25 ml BMS were incubated at 33 °C for 18 h. After washing twice at 4 °C the cells were resuspended in 4 ml BMS and incubated with 400 μCi ^{51}Cr (sodium chromate) at 37 °C for 30 min. The cells were washed four times in cold BMS, held at 4 °C for 1 h, washed again and resuspended at 10^6 cells ml^{-1} in BMS. Mixtures of 1 ml of this cell suspension and 10^7 LWC, LNC or PBL in 1 ml BMS were made in scintillation vials and the vials incubated at 37 °C for 18 h. Each vial was filled with 8 ml cold PBS and then centrifuged at 300g for 10 min. Each supernatant fluid (5 ml) was transferred to a clean vial for gamma counting. The remaining cells were lysed by adding 5 ml 1% sodium dodecyl sulphate in PBS and also counted. The percentage specific release of ^{51}Cr was calculated as follows:

$$\frac{\text{c.p.m. in supernatant} - \text{c.p.m. in medium control supernatant}}{\text{Total c.p.m. in cells} + \text{supernatant} - \text{c.p.m. in medium control supernatant}} \times 100$$

* Hyperimmune gnotobiotic calf Pi-3 virus antiserum, haemagglutination inhibition titre 1:10,000. Used at a final dilution of 1:100.

† Values for calves 1–4 and PBL are based on two replicates. All other values are based on four replicates. Spontaneous ^{51}Cr leakage from uninfected CK cells was 14.4–18.6% and from virus-infected cells was 29.6–32.4%.

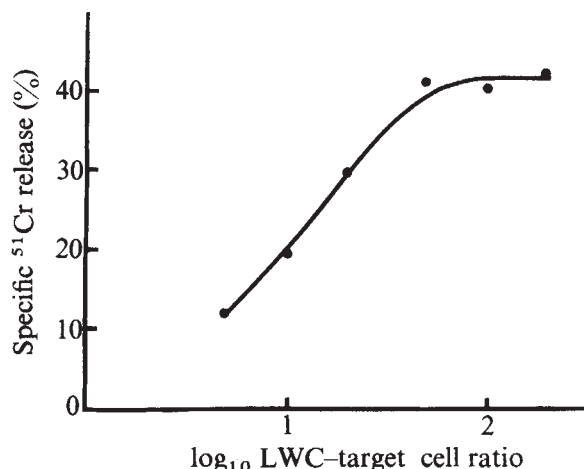


Fig. 1 Effect of different ratios of LWC to target cells on cytotoxicity. Methods are similar to those described in Table 2 except that no antiserum was used and concentration of LWC was varied from 5×10^6 to 2×10^7 cells ml^{-1} . Values are based on three replicates. Spontaneous ⁵¹Cr leakage from uninfected CK cells was 16.7% and from virus-infected cells was 21.3%. LWC were collected from a 9-d-old conventional calf.

a potent cytotoxic activity against virus-infected cells and this activity is inhibited by specific anti-viral serum. In addition, there is no activity against uninfected cells, and therefore exposed viral antigen in the cell membrane seems to be necessary for LWC action. The readily demonstrable cytotoxicity of LWC from two gnotobiotic calves never exposed to Pi-3 virus (16.2% specific ⁵¹Cr release by calf 6, Table 1 and 17% by another control calf tested as in Table 2) shows, however, that the LWC do not require previous experience of Pi-3 virus antigens. Therefore, the LWC activity probably reflects a general reaction against 'foreign' components in the infected target cell and this hypothesis is currently under test.

Various immunological mechanisms have been described for the destruction of virus-infected cells. Complement-

mediated reactions in the presence of antibody^{11,12} or antibody and leukocytes⁵ are unlikely to have occurred in the current work as all sera were heat-inactivated. Other antibody-dependent mechanisms involving various types of effector cell^{11,7,8,13,14} are also ruled out because specific antiserum, even at high dilution, inhibits rather than enhances LWC cytotoxicity. The lysis of virus-infected cells by specifically sensitised T cells^{6,9} is an unlikely explanation of our results because the LWC activity was detected in the absence of specific previous sensitisation of the donor calves, and recent experiments show that only glass-adherent cells are cytotoxic. Supernatant fluid from co-cultures of LWC with target cells is non-cytotoxic, indicating that soluble factors are not involved. Therefore, it is most likely that LWC cytotoxicity is caused by macrophages^{2,15-17} activated following the entry of foreign antigens into the respiratory tract. Preliminary evidence suggests that this macrophage activity increases during the first week of life paralleling the macrophage-mediated, age-dependent resistance of mice to herpes simplex virus^{18,19}.

Pi-3 virus is regarded as a major cause of respiratory disease in calves²⁰ and the destruction of Pi-3 virus-infected cells in the respiratory tract may be an important mechanism for the elimination of virus. The precise role and significance of this phenomenon in the defence of the respiratory tract against virus infection is being investigated.

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- Allison, A. C., *Ann. Inst. Pasteur*, **123**, 585-608 (1972).
- Allison, A. C., *Scientific Basis of Medicine Annual Reviews*, 49-73 (Athlone, London, 1972).
- Allison, A. C., *Transplant. Rev.*, **19**, 3-55 (1974).
- WHO Technical Report Series, No. 519 (1973).
- Lodmell, D. L., Nuva, A., Hayashi, K. and Notkins, A. L., *J. exp. Med.*, **137**, 706-720 (1973).
- McFarland, H. F., *J. Immun.*, **113**, 173-180 (1974).
- Shore, S. L., Nahmias, A. J., Starr, S. E., Wood, P. A., and McFarlin, D. F., *Nature*, **251**, 350-352 (1974).
- Rager-Zisman, B., and Bloom, B. R., *Nature*, **251**, 542-543 (1974).
- Gardner, I., Bowern, N. A., and Blanden, R. V., *Eur. J. Immun.*, **4**, 68-72 (1974).
- Harris, R., and Ukaejiofo, E. O., *Lancet*, **ii**, 327 (1969).
- Brier, A. M., Wohlenberg, C., Rosenthal, J., Mage, M., and Notkins, L., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 3073-3077 (1971).
- Smith, J. W., Adam, E., Melnick, J. L., and Rawls, W. E., *J. Immun.*, **109**, 554-564 (1972).
- Perlmann, P., Perlmann, H., and Wigzell, H., *Transplant. Rev.*, **13**, 91-114 (1972).
- MacLennan, I. C. M., *Transplant. Rev.*, **13**, 67-90 (1972).
- Mackness, G. B., *J. exp. Med.*, **129**, 973-992 (1969).
- Hibbs, J. B., Lambert, L. H., and Remington, J. S., *Proc. Soc. exp. Biol. Med.*, **139**, 1049-1052 (1972).
- Evans, R., and Alexander, P., *Nature*, **236**, 168-170 (1972).
- Zisman, B., Hirsch, M. S., and Allison, A. C., *J. Immun.*, **104**, 1155-1159 (1970).
- Hirsch, M. S., Zisman, B., and Allison, A. C., *J. Immun.*, **104**, 1160-1165 (1970).
- Phillip, J. I. H., and Darbyshire, J. H., *Adv. Vet. Sci. comp. Med.*, **15**, 159-199 (1971).

Table 2 Effect of Pi-3 virus-specific antiserum on calf LWC cytotoxicity for Pi-3 virus-infected CK cells

LWC§	Final antiserum† dilution	% Specific ⁵¹ Cr release* from Pi-3 virus-infected cells
+	—	42.2
+	10 ⁻¹	6.9
+	10 ⁻²	6.6‡
+	10 ⁻³	7.4
+	10 ⁻⁴	29.9‡
+	10 ⁻⁵	41.1
—	—	0
—	10 ⁻¹	0
—	10 ⁻³	0
—	10 ⁻⁵	0

Secondary CK cells were suspended in either Pi-3 virus (50 PFU per cell) or medium, mixed with ⁵¹Cr (40 μCi per 10^6 cells) and incubated at 37 °C for 1 h. The cells were then washed four times in cold BMS, resuspended at 10^6 cells ml^{-1} and dispensed into scintillation vials (1 ml per vial). After incubation at 37 °C for 18 h the cells in the vials were washed twice with cold BMS. One ml of BMS containing 10^7 LWC and the relevant antiserum dilution was then added. After incubating at 37 °C for 18 h the vials were treated as described in Table 1.

* Values are based on three replicates. Spontaneous ⁵¹Cr leakage from uninfected CK cells was 15.9-17.8% and from virus-infected cells was 21.7-23.9%.

† Hyperimmune gnotobiotic calf antiserum as in Table 1.

‡ Mean value of spontaneous ⁵¹Cr leakage from Pi-3 virus-infected control cells used to calculate these figures.

§ LWC were collected from a 6-d-old conventional calf.

Cell-mediated immunity to hapten modified self- and non-self antigens

CELL-mediated lysis of cells infected by certain viruses or coupled with haptenic groups has been reported to occur only in situations in which the target cell and cytotoxic effector cell are either syngeneic or semi-allogeneic and cannot be observed when they are allogeneic¹⁻⁴. Thus, spleen cells from random-bred chickens bearing Rous sarcomas are very cytotoxic to autochthonous tumour cells but show little cytotoxicity on allogeneic tumour cells. Mouse cells infected with lymphocytic choriomeningitis virus (LCM) are only lysed by sensitised thymus-derived cells (T cells) syngeneic or semi-allogeneic to the target cells⁵. Similarly, spleen cells

sensitised *in vitro* to trinitrophenyl (TNP) coupled syngeneic spleen cells were reported to lyse only syngeneic but not allogeneic hapten-coupled target cells³. Since in all these models the induction of cell-mediated cytotoxicity was carried out in a syngeneic situation, it is clear that the phenomenon is not a result of the induction step, but is mediated through the effector step of cell-mediated cytotoxicity. It was concluded from these results that the cytotoxic effector cells recognise the modified self antigen²⁻⁴ rather than the determinants introduced into the cell membrane (the virus or the hapten). I report here, however, results which show that TNP-specific cytotoxicity mediated by cytotoxic T cells (T killer cells) can be detected under appropriate assay conditions.

It was recently reported⁵ that T killer cells able to lyse hapten-coupled target cells can be induced *in vitro* by both syngeneic and allogeneic hapten-coupled cells and that the activity of such spleen cells can be assayed on hapten-coupled targets either syngeneic or allogeneic to the immunising cells. I have chosen this model to reinvestigate the question of whether T killer cells can in fact recognise antigenic determinants artificially introduced into the cell membrane. If they can, then competitive inhibition of the cytotoxic activity should be possible using free antigenic determinants.

C57BL/6 spleen cells were sensitised (*in vitro*) to TNP-coupled syngeneic spleen cells or TNP-coupled allogeneic DBA/2 P815 mastocytoma cells (TNP-P815)⁵. On day 5 the cultures were collected and assayed for cytotoxicity on TNP-coupled C57BL/6 leukaemia EL4 (TNP-EL4) or TNP-coupled BALB/c lymphoma S49 (TNP-S49) targets⁵. Inhibition studies were performed using TNP-coupled chicken erythrocytes (TNP-CRBC) as inhibitors⁶, since free hapten (TNP-lysine) does not inhibit killing of TNP-coupled targets in this model system^{3,5}. This is not surprising since purified H2 histocompatibility antigens⁸, in contrast to whole cells⁷, do not inhibit cytotoxicity of allogeneically sensitised T cells. The cytotoxicity of C57BL/6 spleen cells sensitised to syngeneic TNP-coupled spleen cells cannot be inhibited

by TNP-CRBC if the assay is done on TNP-EL4 targets (Fig. 1). Cytotoxicity of the same spleen cells, however, is inhibited by TNP-CRBC if cytotoxicity is assayed on allogeneic TNP-S49 cells. Similarly, if C57BL/6 spleen cells are sensitised to TNP-P815 and assayed on TNP-EL4, the cytotoxicity observed is again inhibitable by TNP-CRBC. This would suggest that TNP-specific cytotoxicity is only observed if the hapten-coupled cell used as target is allogeneic to the hapten-coupled sensitising cell. Two explanations can be considered: (1) within the total population of cytotoxic cells there are two distinct populations—one recognises TNP on allogeneic target cells and is inhibitable by TNP-CRBC, the other recognises modified self antigens and is not inhibitable by TNP-CRBC; (2) the population of cells which recognises modified self also recognises TNP, but the affinity of their receptors for TNP is low. Although the experiment presented cannot distinguish between these alternatives, both of them account for the observation that cell-mediated cytotoxicity to hapten or virus-modified cells is higher if the assay is done on targets which share histocompatibility antigens with the sensitising immunogen¹⁻³. It seems, therefore, that a preferential sensitisation to TNP-modified self antigens occurs (refs 3 and 5, and Fig. 1). TNP-specific cytotoxicity, however, can be detected under appropriate assay conditions. This supports and extends previously published data showing that T cells can be sensitised to haptenic groups⁹⁻¹¹.

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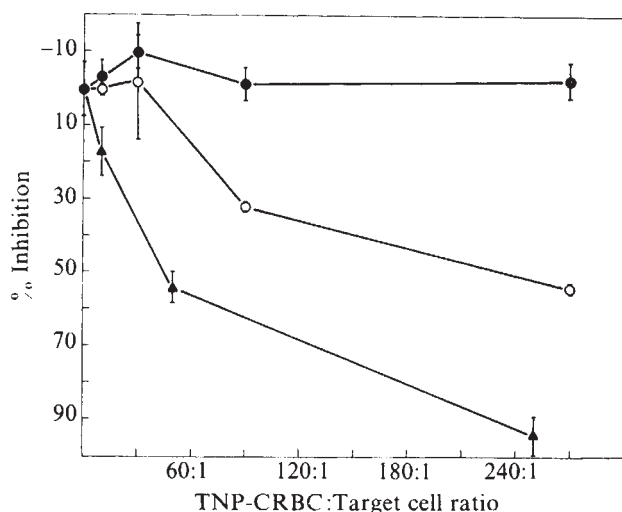
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- ¹ Wainberg, M. A., Markson, Y., Weiss, D. W., and Doljanski, F., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3565-3569 (1974).
- ² Doherty, P. C., and Zinkernagel, R. M., *J. Immun.*, **114**, 30-33 (1975).
- ³ Shearer, G. M., *Eur. J. Immun.*, **4**, 527-533 (1974).
- ⁴ Zinkernagel, R. M., and Doherty, P. C., *Nature*, **251**, 547-548 (1974).
- ⁵ Dennert, G., and Hatlen, L. E., *J. Immun.* (in the press).
- ⁶ Todd, R. F., Stulting, R. D., and Amos, D. B., *J. cell. Immun.* (in the press).
- ⁷ Lesley, J., Hyman, R., and Dennert, G., *J. natn. Cancer Inst.*, **53**, 1759-1765 (1974).
- ⁸ Iverson, G. M., *Nature*, **227**, 273-274 (1970).
- ⁹ Mitchison, N. A., *Eur. J. Immun.*, **1**, 18-27 (1971).
- ¹⁰ Rubin, B., and Wigzell, H., *Nature*, **242**, 467-469 (1973).
- ¹¹ Möller, E., *Scand. J. Immun.*, **3**, 339-355 (1974).

Fig. 1 C57BL/6 spleen cells were sensitised to TNP-coupled C57BL/6 spleen cells and TNP-P815 (ref. 5) and assayed for cytotoxicity on normal and TNP-coupled target cells at an attacker-to-target cell ratio of 100:1 (ref. 5). TNP-CRBC (ref. 5) were added as inhibitors at the different ratios shown on the abscissa. The time points were taken after 4 h (●) and 8 h (○, ▲) respectively. The percentage inhibition of the net cytotoxicity on TNP-EL4 as inhibited by TNP-CRBC is also plotted, with the s.e.m. values shown as vertical bars. Cytotoxicity was 51.3% on TNP-EL4 and 0% on EL4 for spleen cells sensitised to TNP-C57BL/6 spleen (●); 9.4% on TNP-S49; and 0% on S49 for the same spleen cells (○), 22.2% on TNP-EL4 and 5.6% on EL4 for spleen cells sensitised to TNP-P815 (▲). There was no inhibition at all by uncoupled CRBC.



Comparative growth of bovine lymphosarcoma cells and lymphoid cells infected with *Theileria parva* in athymic (nude) mice

TUMOUR-LIKE growth of bovine lymphoid cells infected with *Theileria parva* has been observed in whole-body irradiated athymic (nude) mice using cells isolated direct from fatal cases of East Coast fever¹, and also using lymphoid cells infected with *T. parva*, maintained as lines in culture². Here we compare the growth, in irradiated athymic mice, of this latter cell type with that of similar bovine transformed lymphoid cells of known neoplastic origin. The results contrast with those recorded in the natural conditions in cattle, since cells infected with *T. parva* grew malignantly in mice whereas bovine lymphosarcoma cells formed localised non-invasive tumours.

Twelve 6-8-week-old male athymic (nude) mice (Carworth, Huntingdon) were irradiated at 700 rad from a ⁶⁰Co source. Twelve similar mice were untreated. Four mice from each group were inoculated subcutaneously with bovine lymphoid (C2) cells infected with *T. parva*³; four from each group with bovine lymphosarcoma (BL3) cells, and four from each group with normal bovine lymphoid cells.

Tumours or tumour-like masses developed in all the irradiated

Table 1 Comparative growth of bovine lymphosarcoma (BL3) cells and (C2) cells infected with *T. parva* in athymic mice

Cell-host reaction	BL3 cells	C2 cells
Growth in athymic mice		
Non-irradiated	None	Occasional limited growth
Irradiated	Very good. No regression observed	Very good. Occasional regression with recovery
Growth first detectable	Days 4-7	Days 2-3
Nature of growth	No infiltration of cells, growth localised at site of inoculation. No ulceration only slight necrosis of tumour	Extensive infiltration and destruction of local musculature. Ulceration and necrosis of tumour common, sometimes extensive
Cellular dissemination	None detected on histology or organ impression smears	Parasitised cells commonly seen in blood, lung and liver; also in kidney, spleen, brain and lymph nodes
Clinical symptoms in host	No generalised symptoms	Debility, wasting, terminal respiratory distress and anorexia
Host death	Not recorded	Common 30-50 d
Passage of tumour cells to athymic mice		
Non-irradiated	None	Occasional limited growth
Irradiated	About 80% take	About 80% take

The details recorded represent the collected observations from 11 mice (BL3 cells) and 50 mice (C2) cells. BL3 cells were obtained from a fatal case of lymphosarcoma⁸ in an adolescent steer (Code 8004) and were supplied by the Cell Culture Laboratory, Naval Biomedical Research Laboratory, Oakland, California at passage 1. A pure lymphoblastoid cell suspension was isolated in this laboratory, and inoculated into mice at passage 10. C2 cells were passage 24. Both cell lines were grown as described previously³ and each mouse received 5×10^7 cells in 0.2 ml medium from day 2 static cultures. Control mice received similar inocula of normal bovine lymphoid cells isolated by the method of Thorsby and Bratlie⁹ from 100 ml of normal bovine blood collected by venipuncture into 50 IU heparin ml⁻¹. Inoculations were given approximately 1 h after collection of blood.

mice inoculated with C2 or BL3 cells, but not in any other groups. The growth of C2 cells and the responses of the host were similar to those described previously^{1,2}. In the irradiated mice inoculated with BL3 cells, tumours were first detected at the site of inoculation on day 7; they continued to grow as localised masses and became very large. There was no evidence of vascular spread of cells and mice remained clinically normal until the tumours became so large that they hampered movement and inhibited feeding and drinking. This stage was reached between days 50 and 70, and mice were then destroyed on humane grounds. BL3 cells were passaged directly to further athymic mice as described previously¹, and a similar growth pattern was recorded: tumours occurring in irradiated mice, but not in untreated mice.

Table 1 summarises the different growth patterns of C2 and BL3 cells in athymic mice, recorded in the above and other experiments. BL3 cells formed discrete circumscribed tumours with no evidence of metastasis, whereas C2 cells infiltrated extensively causing a malignant neoplastic condition. These findings contrast with the natural conditions in cattle—lymphosarcoma being a malignant neoplasia—and East Coast fever, a non-malignant condition with no evidence of tumour formation.

C2 and BL3 cells grew consistently only in irradiated athymic mice, suggesting that non-irradiated mice retained some cell-mediated immune activity which was abolished by irradiation. Residual T cell activity in athymic mice has been demonstrated⁴⁻⁶, and the present work further indicates the functional nature of these cells in non-irradiated athymic mice. Because of its nonspecific action, however, irradiation also suppresses B cell activity and thus antibody production. This may be significant in the present context since anti-tumour antibodies have recently been demonstrated in the sera of athymic mice⁷. Irradiation would presumably inhibit production of such antibodies; anti-tumour activity could then be abolished if sufficient tumour antigens were inoculated to eliminate circulating antibodies.

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¹ Irvin, A. D., Stagg, D. A., Kanhai, G. K., and Brown, C. G. D., *Nature*, **253**, 549-550 (1975).

² Irvin, A. D., thesis, R. Coll. Vet. Surgeons, London (1975).

³ Malmquist, W. A., Nyindo, M. B. A., and Brown, C. G. D., *Trop. anim. Hlth Prod.*, **2**, 139-145 (1970).

⁴ Raff, M. C., *Nature*, **246**, 350-351 (1973).

⁵ Loor, F., and Kindred, B., *J. exp. Med.*, **138**, 1044-1055 (1973).

⁶ Loor, F., and Roelants, G. E., *Nature*, **251**, 229-230 (1974).

⁷ Martin, W. J., and Martin, S. E., *Nature*, **249**, 564-565 (1974).

⁸ Theilen, G. H., et al., *J. natn. Cancer Inst.*, **40**, 737-749 (1968).

⁹ Thorsby, E., and Bratlie, A., in *Histocompatibility Testing* (edit. by Terasaki, P. I.), 655 (Munksgaard, Copenhagen, 1970).

Nonspecific cross reacting antigen as a marker for human polymorphs, macrophages and monocytes

WE have described¹ an antigen able to cross react with the carcinoembryonic antigen of the digestive system (CEA) and named it nonspecific cross reacting antigen (NCA) because of its reaction with CEA and its lack of tissue and cancer specificity. NCA was found in perchloric extracts of gastrointestinal tumours and in lesser amounts in those of non-cancerous mucosa, lung and spleen. The same antigen has been described under different names (NGP, CCEA II) in other laboratories^{2,3}. We pointed out that NCA bore a specific antigenic determinant not shared by CEA¹.

This has provided us with the possibility of raising a

specific rabbit anti-NCA antiserum, and of using it to study the localisation of NCA by indirect immunofluorescence the second layer being a fluoresceinated sheep anti-rabbit globulin antiserum (Institut Pasteur, Paris). We have already shown the presence of NCA at the apical pole of glandular cells of gastrointestinal tumours and non-cancerous colonic mucosa⁴. When studying other organs, we found that NCA is a normal component of polymorphs and of mononuclear cells looking like macrophages and monocytes. Similar observations have been made independently¹⁰.

When examining frozen sections of human lung, we observed that many cells scattered in the alveolar walls were positively stained with anti-NCA antiserum. We therefore studied fixed cultured alveolar macrophages and found that they reacted strongly with the same antiserum, showing a cytoplasmic fluorescence, either diffuse or in the form of coarse granules.

On spleen frozen sections many cells were positive with anti-NCA antiserum, almost all of them being outside the lymphoid follicles. Spleen imprints enabled a better characterisation of the fluorescent cells: most of them were polymorphs, some were large mononuclear cells (Fig. 1).

While blood cell smears (the red cells had been lysed by hypotonic ammonium chloride) showed many positive cells. As on the spleen imprints, almost all the polymorphs were fluorescent, together with some large mononuclear cells. Small lymphocytes were always negative. The specificity of the reaction was assessed using NCA-absorbed antiserum, staining only eosinophils, which nonspecifically bind any fluorescent globulin.

The positive mononuclear cells were more numerous on smears made with the lymphocyte layer obtained after centrifugation of the blood on Ficoll-Trisil which enabled more careful study. Most of these cells were large, the same size or larger than polymorphs, with large nuclei, sometimes eccentric, and often slightly incurvated. Their fairly abundant cytoplasm gave a fluorescence which varied in intensity, and was generally diffuse or finely granular. Its tinctorial properties could not be determined precisely, as the Giemsa reagent did not stain the cytoplasm after fluorescent labelling.

To identify these NCA-positive mononuclear cells, we used different ways: Adherence to glass wool columns was uneven, some cells being able to bind the column and others not. In contrast, all cells fixed to nylon wool columns. They were not able to phagocytose iron carbonyl particles, nor aggregated γ -globulins conjugated to fluorescein (in this latter case, after the phagocytosis step, the cells were fixed with ethanol, then labelled with rabbit anti-NCA antiserum and Rhodamin-conjugated anti-rabbit globulin. Many polymorphs were observed containing green granules in a diffusely red cytoplasm; mononuclear cells were only red). EA rosettes were prepared, recovered by centrifugation on Ficoll-Trisil, and smeared. Among the many mononuclear cells, a part was stained by anti-NCA antiserum. The positive cells were much more numerous than on ordinary Ficoll-Trisil smears, indicating that NCA-positive cells were engaged in EA rosetting and concentrated by this procedure, and therefore had Fc receptors on their surface. We looked for intracytoplasmic immunoglobulins using double fluorescence on Ficoll-Trisil and EA rosette smears, and concluded that many, if not all, NCA-positive mononuclear cells did not contain detectable immunoglobulins and that Ig-positive cells were negative for NCA.

It is thus likely on morphological and physiological grounds that NCA-positive mononuclear cells belong to the monocyte series, and that they could be considered as ancestors of the macrophages found in lung alveolar walls. They do not, however, have all the characteristics classically attributed to monocytes as they lack phagocytic activity. Yet it is quite possible that young monocytes also do not

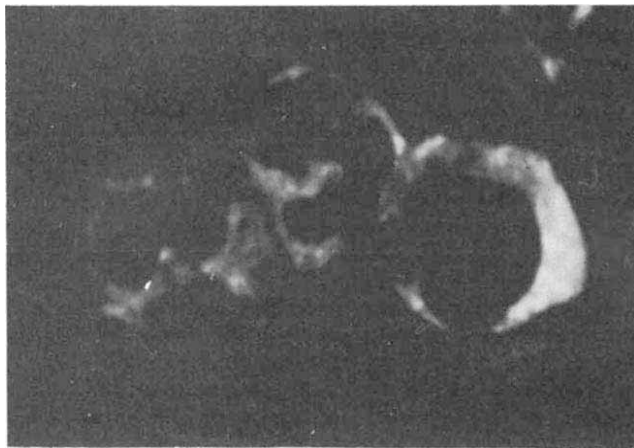


Fig. 1 Spleen imprint: three cells, two polymorphs and a large mononuclear cell, stained with anti-NCA antiserum.

have all these properties. Note that mouse K cells have been described as young monocytes⁵, without phagocytic activity. Yet human K cells differ from NCA-positive cells as they do not adhere to nylon columns⁶. Definitive identification of NCA-positive cells could be obtained by electron microscopy, using, for example, peroxidase-labelled antibodies.

NCA-containing cells were also observed in bone marrow smears, and belonged mainly to the myeloid series; leukaemic myeloblasts were also positive. In ascitic and pleural fluids, polymorphs and large mononuclear cells were also fluorescent.

NCA has thus been found in the cytoplasm of polymorphs and large mononuclear cells. Its subcellular localisation is still to be determined, but it is tempting to speculate that this antigen could belong to the lysosomal system. If so, NCA could equally well be a component of the lysosomal wall as an intralysosomal enzyme. The high carbohydrate content of the NCA molecule makes the first hypothesis more likely. Here again, electron microscopic studies using peroxidase-labelled antibodies could be helpful in determining precisely the subcellular localisation of NCA.

NCA was also found on the surface of polymorphs and large mononuclear cells. This was shown in two ways: membrane fluorescence on living cells, which gave brighter images on polymorphs than on mononuclear cells, and agglutination of the white cells by anti-NCA antiserum. When diluted antiserum was used, small agglutinates were obtained, which could be smeared and stained by Giemsa reagent: they contained mainly polymorphs, and large mononuclear cells looking like monocytes. NCA-absorbed antiserum did not agglutinate the cells, nor stain their surface. Thus the specificity of the reaction could be assessed.

As mentioned above, there is an antigen common to polymorphs and mononuclear cells, which are probably monocytes, that is absent from other white blood cells. This result fits well with the experiments of Metcalf⁷, and Morley *et al.*⁸ showing that there is in the bone marrow an ancestor cell common to polymorphs on one side, and macrophages and monocytes on the other side, the remaining white cells being derived from different stem cell lines.

In conclusion, we have shown that NCA is present in the cytoplasm and on the surface of human polymorphs and monocytes, and in the cytoplasm of macrophages. We believe that this is the first time that a surface marker has been described for human polymorphs. The role of this new marker in characterising normal and pathological human white cells will be studied further. It can for instance be

compared with the chloroacid esterase method⁹ known to be very useful in the diagnosis of myeloblastic leukaemias.

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- ¹ Von Kleist, S., Chavanel, G., and Burtin, P., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 2492-2494 (1972).
- ² Mach, J. P., and Pusztaszeri, G., *Immunochemistry*, **9**, 1031-1034 (1972).
- ³ Tuberville, C., Darcy, D. A., Laurence, D., Johns, E. W., and Neville, A. M., *Immunochemistry*, **12**, 841-844 (1973).
- ⁴ Burtin, P., Von Kleist, S., Sabine, M. C., and King, M., *Cancer Res.*, **33**, 3299-3305 (1973).
- ⁵ Greenberg, A. M., Shen, L., and Roitt, I. M., *Clin. exp. Immunol.*, **15**, 251-259 (1973).
- ⁶ Wisloff, F., and Froland, S., *Scand. J. Immun.*, **2**, 151-157 (1973).
- ⁷ Metcalf, D., *J. Cell. Physiol.*, **77**, 277-280 (1971).
- ⁸ Morley, A., Richard, K., Howard, D., and Stohlmann, F., *Blood*, **37**, 14-22 (1971).
- ⁹ Burstone, M., *J. natn. Cancer Inst.*, **12**, 167-173 (1957).
- ¹⁰ Bordes, M., Knobel, S., Martin, F., *Eur. J. Cancer* (in the press).

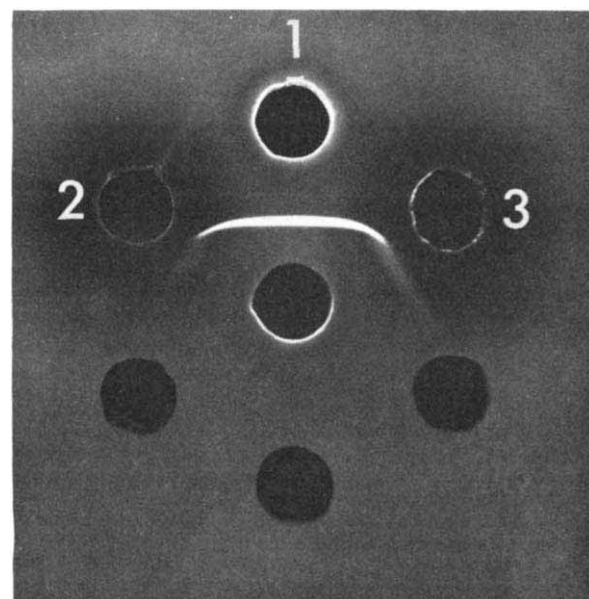


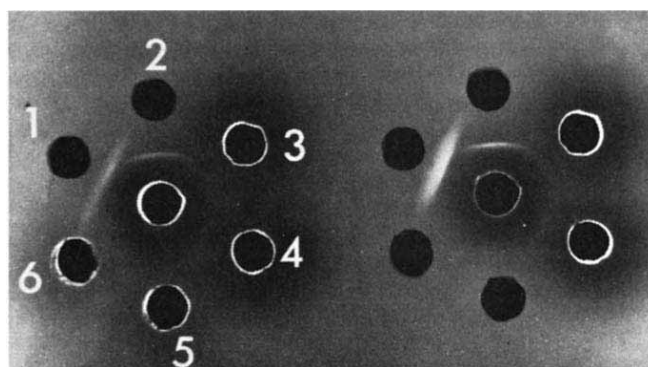
Fig. 1 Immunodiffusion in agarose. 1, Human foetal serum; 2, AFP-positive serum from a hepatoma-bearing *Cercopithecus* monkey; 3, a similar serum from a *Macaca* monkey; centre well, sheep anti-human AFP.

Further evidence that the antibodies produced by the monkeys immunised with rabbit AFP also reacted with their own AFP, was provided by experiments showing that these antisera lacked free AFP. Using immunoabsorbents it is possible to isolate small amounts of AFP from normal monkey sera, whereas no detectable AFP was recovered from the immune sera (Table 2).

We have thus succeeded in producing antibodies to autologous AFP in monkeys. This is in accordance with similar data obtained in rabbits⁵ and mice (E. Ruoslahti and H. Pihko, unpublished). It has recently become evident that many other oncofoetal antigens by themselves may cause an immune response in multiparous animals or tumour bearers. Such antigens characteristically behave as group specific antigens, that is, a common antigen is shared by tumours arising from a given cell type¹²⁻¹⁴. Human tumour antigens demonstrable by cytotoxicity of autologous lymphocytes show similar tissue specificity¹⁵ and it has been suggested that they represent foetal antigens. Possible immunogenicity of foetal antigens may thus be of considerable importance in tumour immune reactions.

Why AFP is not immunogenic by itself in its host of origin may be explained by the extremely high foetal levels

Fig. 2 Immunodiffusion in agarose. Left: anti-AFP from *Cercopithecus*; right: anti-AFP from *Macaca*. 1, Purified rabbit AFP; 2, purified human AFP; 3, AFP-positive *Cercopithecus* serum; 4, AFP-positive *Macaca* serum; 5, normal *Cercopithecus* serum; 6, normal *Macaca* serum.



Breakage of tolerance to α foetoprotein in monkeys

α FOETOPROTEIN (AFP) is a foetal protein produced in large quantities by the foetal liver, yolk sac, and certain tumours such as hepatomas and teratomas^{1,2}. AFP does not cause immune response in a tumour-bearing host or in situations in which a transient increase in AFP takes place³. This apparent tolerance, however, can be broken in rabbits or mice by immunisation with heterologous⁴ or chemically modified homologous AFP (ref. 5). Both these approaches have been found effective in breaking tolerance to other antigens⁶. It has been shown that AFP-producing hepatoma cells are killed by anti-AFP antibodies *in vitro*^{7,8}. AFP may thus be of potential use as target antigen in tumour immunotherapy.

We show here that tolerance to autologous AFP can be broken in monkeys by immunisation with rabbit AFP. It is likely that the same procedure could be used in humans should animal experiments warrant such an approach to immunotherapy of AFP-producing human tumours.

Our choice of rabbit AFP for immunisation of monkeys was based on the observation that human (and monkey) AFP shows strong immunological cross reactivity with rabbit AFP when sheep antisera are used. Two monkeys, one *Cercopithecus ethiops* (Vervet) and one *Macaca irus* (Cynomolgus), were immunised with purified rabbit AFP (ref. 9). Three injections of 0.5 mg AFP in Freund's complete adjuvant were given 2 weeks apart and the animals were bled 2 weeks after the last injection. Antibody response against AFP was measured by immunodiffusion and/or radioimmunoassay¹⁰. In the latter test we used human or rabbit AFP as the labelled antigen. This is justified in view of the striking immunological similarity of human AFP and AFP from the monkeys used (Fig. 1). Concentration of AFP from monkey sera was achieved by adsorption to and elution from sheep anti-human AFP immunoabsorbents⁹.

Both monkeys produced strong precipitating antibodies against rabbit AFP (Fig. 2) and the antisera also reacted with purified human AFP and the *Cercopithecus* and *Macaca* sera containing AFP, but not in a detectable manner with normal sera from the same species. High binding titres were obtained with rabbit and human AFP in radioimmunoassay with the majority of antibodies directed against the immunogen (Table 1). As seen in Fig. 2, the monkey anti-AFP sera react with lines of identity against homologous monkey AFPs as well as against human AFP.

Table 1 Binding of ^{125}I -labelled rabbit and human AFP by monkey anti-rabbit AFP sera

Serum	Titre* with rabbit AFP	Titre* with human AFP
<i>Cercopithecus</i> anti-rabbit AFP	9×10^4	9×10^3
<i>Macaca</i> anti-rabbit AFP	3.5×10^4	3×10^3

* Reciprocal of serum dilution binding 50% of the label precipitable at antibody excess.

(up to several mg ml^{-1}) and the residual small concentrations in adult serum (a few ng ml^{-1} in humans^{10,11}, about 100 ng ml^{-1} in mice¹⁶ and rabbits⁵).

It is likely that these AFP concentrations are much higher than is the case for those oncofoetal antigens which cause an immune response in adult animals. These latter antigens may also only be expressed during an earlier embryonic period than AFP, thus allowing more complete loss of immune tolerance¹⁷.

AFP is, unlike most other oncofoetal antigens, a well characterised molecule. Several animal models are available for studies, thus enabling accurate measurement of various aspects of AFP expression and manipulation of its immunogenicity. Recent results from several laboratories show that in spite of the fact that AFP is secreted by hepatoma cells, such cells can be killed using heterologous anti-AFP (refs 7 and 8). The limited attempts to show tumour resistance in animals treated with anti-AFP or immunised against their own AFP have given inconclusive results^{4,8}, but definitive degenerative changes have been

Table 2 Radioimmunoassay of AFP isolated from normal and anti-rabbit AFP monkey sera using immunoabsorbents*

Sample	Amount of AFP (ng):	
	Original sample	Eluate
<i>Cercopithecus</i> Normal	25	10.0
Anti-rabbit AFP	—†	<1.0
<i>Macaca</i> Normal	74	10.3
Anti-rabbit AFP	—†	<1.0

* A 25 ml sample of monkey serum was treated with 18% sodium sulphate to remove gammaglobulin and the supernatant was incubated for 3 d with 50 μl Sepharose conjugated anti-human AFP. The particles were eluted with 8M urea and analysed for AFP in radioimmunoassay^{5,8}.

† The original AFP content of anti-rabbit AFP sera could not be measured because of interference in the assay by the residual anti-AFP antibody present in the sodium sulphate supernatant.

demonstrated in the liver of foetuses from rats immunised against their own AFP (ref. 18). These results suggest that further studies on breakage of tolerance to AFP and the effect of the induced antibodies on the appearance and growth of AFP-producing tumours may prove rewarding.

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¹ Abelev, G. I., *Transplant. Rev.*, **20**, 3–37 (1974).

² Ruoslahti, E., Pihko, H., and Seppälä, M., *Transplant. Rev.*, **20**, 38–60 (1974).

³ Nishi, S., Watabe, H., and Hirai, H., *J. Immun.*, **109**, 957–960 (1972).

⁴ Goussev, A., and Yasova, A., in *Colloques L'INSERM L'Alphafetoprotéine* (edit. by Masseyeff, R.), 255–270 (1974).

⁵ Ruoslahti, E., Pihko, H., Becker, M., and Mäkelä, O., *Eur. J. Immun.*, **5**, 7–10 (1975).

⁶ Weigle, W. O., Chiller, J. M., and Habicht, G., in *Progress in Immunology* (edit. by Amos, B.), 311 (Academic, New York and London, 1971).

⁷ Tsukada, Y., Mikuni, M., Watabe, H., Nishi, S., and Hirai, H., *Int. J. Cancer*, **13**, 187–195 (1974).

⁸ Mizejewski, G., and Allen, R. P., *Nature*, **250**, 50–52 (1974).

⁹ Pihko, H., Lindgren, J., and Ruoslahti, E., *Immunochimistry*, **10**, 381–385 (1973).

¹⁰ Ruoslahti, E., and Seppälä, M., *Int. J. Cancer*, **8**, 374–383 (1971).

¹¹ Ruoslahti, E., and Seppälä, M., *Nature*, **235**, 161–162 (1972).

¹² Baldwin, R. W., Graves, D., and Vose, B. M., *Int. J. Cancer*, **13**, 135–142 (1974).

¹³ Steele, G., and Sjögren, H. O., *Int. J. Cancer*, **14**, 435–444 (1974).

¹⁴ Hellström, I., and Hellström, K. E., *Int. J. Cancer*, **15**, 1–16 (1975).

¹⁵ Hellström, K. E., and Hellström, I., *Adv. Immun.*, **18**, 209–277 (1974).

¹⁶ Pihko, H., and Ruoslahti, E., *Int. J. Cancer*, **12**, 354–360 (1973).

¹⁷ Alexander, P., *Nature*, **235**, 137 (1972).

¹⁸ Nishi, S., Watabe, H., and Hirai, H., *Tumor. Res.*, **8**, 17–22 (1973).

Localisation of caps on mouse B lymphocytes by scanning electron microscopy

WE have used cathodoluminescence (CL) to detect fluorescein-labelled anti-IgG-induced caps on mouse B lymphocytes by means of the scanning electron microscope (SEM). This approach, which has as yet been little explored, depends critically on efficient collection of a weak CL signal^{1,2}. It

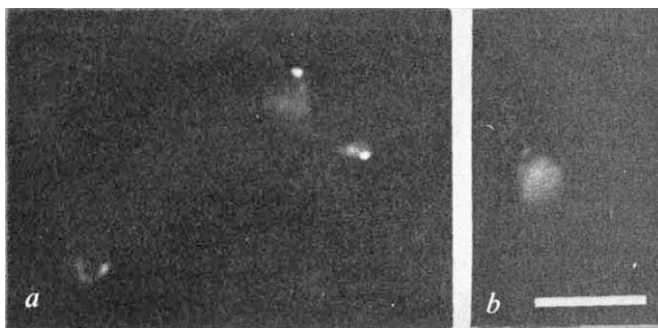


Fig. 1 Fluorescence micrograph of capped mouse lymphocytes obtained after incubation with fluorescein-labelled goat anti-mouse IgG. Bar = 10 μm . *a*, Unfixed; *b*, after fixation in glutaraldehyde, dehydration and critical point drying. Unfixed cells from lymph nodes of C3H mice were incubated with fluorescein-labelled goat anti-mouse IgG (Hyland) at 37 °C for 20 min. Some of the cells were then examined in an ultra-violet microscope to determine if they had acquired caps. The cells were then fixed in 2.5% buffered glutaraldehyde for 30 min, washed and dehydrated for 10 min in each of the following: 30%, 50%, 70%, 95% and 100% ethyl alcohol. Indexed copper grids (mesh 200), coated with Formvar and a light layer of carbon were then placed on top of a Millipore filter (FLG PO 1300, porosity 0.25 μm). The cell suspension in 100% alcohol was then passed through the filter so that some of the lymphocytes settled on the grids and others on the filter. Since Formvar and carbon coated grids showed no fluorescence, the cells on the grids could then be examined for CL as well as in the secondary electron mode. Cells on the filter could only be examined by the secondary electron mode because of the auto-fluorescence of the filter. A holder containing the Millipore filter and grids, with adherent lymphocytes, wet in absolute alcohol, was transferred to a critical point dryer (SAMDR1 TM PVT-3). Liquid CO_2 was then introduced slowly into the critical point dryer chamber, and left flowing through the chamber for 10–15 min to remove alcohol. The temperature of the chamber was then raised and when it reached 40 °C, the pressure was decreased at a rate of 100 pounds min^{-1} . The grids were removed and mounted on special stubs with Aquadag, for examination in the SEM (Stereoscan Mark II fitted with a sensitive CL detector system³). The special stubs had been drilled with holes of diameter 2 mm, painted inside with Aquadag, and the grids were mounted over those holes, to reduce CL emission from the stub material underlying the specimen. The SEM was operated at 20 kV with a beam current of 150 μA . After the uncoated lymphocytes had been examined in the secondary electron mode and for CL, the same cells were then coated with gold and examined again in the secondary electron mode. Since gold coating tends to absorb emitted light, the best CL micrographs were obtained on the uncoated samples. The images obtained by CL could be compared with secondary electron micrographs taken on the same cells before and after coating with gold. Polaroid film (type 42) was used at exposure times of 40 s for all three modes.

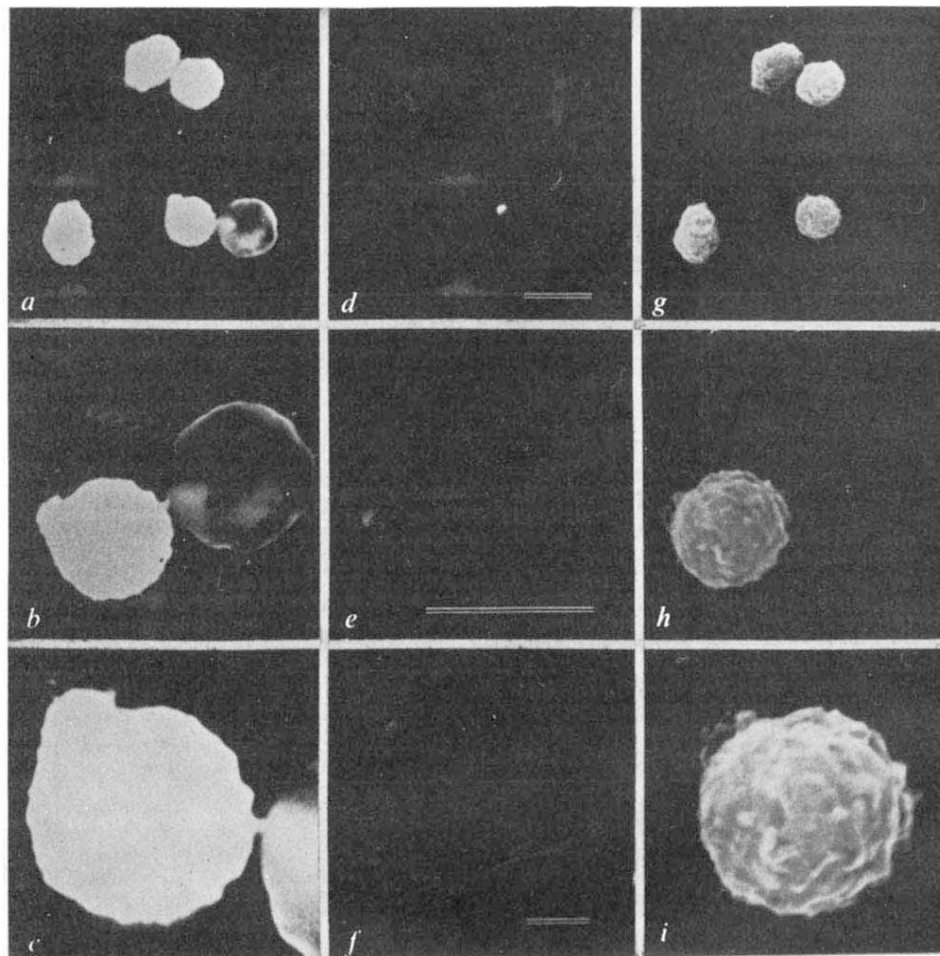


Fig. 2 Electron micrographs of the same capped lymphocyte at different magnifications. *a-c*, Uncoated, secondary electron image; *d-f*, uncoated, cathodoluminescence; *g-i*, gold coated, secondary electron image. Bar = 5 μ m for *d* and *e*, 1 μ m for *f*. *a* and *g* at same magnification as *d*, *b* and *h* as *e*, *c* and *i* as *f*.

provides a means of identifying antigenic markers in the SEM.

Ultraviolet microscopy showed that 20% of unfixed cells had acquired caps in our incubation conditions (Fig. 1*a*). The caps remained visible after fixation in glutaraldehyde and critical point drying (Fig. 1*b*). The same preparation examined by SEM using CL showed a similar proportion of capped cells. In CL micrographs (Fig. 2*d-f*), the cap appeared as a bright semicircular fluorescent region. At lower magnifications (up to $\times 2,000$) the caps appeared brighter and faded more slowly than at the higher magnifications ($\times 5,000$ or $\times 10,000$).

In secondary electron images (Fig. 2*a-c*) the surface features of the uncoated cells were not clear. When the same cells were coated and then examined, however, ridges and microvilli could be seen on their surface (Fig. 2*g-i*). The cap region of the lymphocytes, when examined in greater detail at higher magnifications, appeared as a protrusion, or bulge, on one side of the cell (Fig. 2*i*). This view of the cap agrees with observations made from electron micrographs of sections through the capped region located by ferritin and ^{125}I -labelled anti-Ig antibodies^{3,4}. The cell surface in the cap region also showed several microvilli.

Thus CL can localise caps on mouse B lymphocytes under the SEM. Without the accompanying CL image it was not possible to distinguish cap areas with certainty. This technique enables immunological investigation to be made in the SEM similar to that carried out by ultraviolet fluorescence microscopy. Moreover, the same reagents—fluorescein-labelled antibodies—can be used in both techniques.

Revel *et al.* have shown that haemocyanin can be recognised in the SEM and perhaps ferritin also^{5,6}. These markers and latex particles⁷ can, therefore, be useful for labelling antibodies to localise antigens by means of the SEM. Our study suggests that fluorescein-labelled antibody may also prove a

useful immunological marker for demonstrating surface antigens in cell preparation with the SEM using CL. Our procedure can, in principle, extend the limits of resolution beyond those obtainable with the ultraviolet microscope. Fading of the CL image at magnifications of $\times 10,000$ or above seems to be the chief factor limiting CL resolution. The resolution we obtained in the CL mode was comparable with that obtained by ultraviolet microscopy. If antibody could be labelled so as to give more intense or stable CL than is possible with fluorescein isothiocyanate, resolution in the CL image may be improved.

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¹ Bond, E., Beresford, D., and Haggis, G. H., *J. Microsc.*, **100**, 271–282 (1974).

² Broecker, W., Schmidt, E. H., Pfefferkorn, G., and Beller, F. K., *Eighth Scanning Electron Microscope Symposium*, 243–250 (Illinois Institute of Technology Research Institute, 1975).

³ Unanue, E. R., Perkins, W. D., and Karnovsky, M. J., *J. exp. Med.*, **136**, 885–906 (1972).

- ⁴ Stackpole, C. W., Jacobson, J. B., and Lardis, M. P., *Nature*, **248**, 232-234 (1974).
⁵ Brown, S., Teplitz, M., and Revel, J. P., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 464-468 (1974).
⁶ Revel, J. P., *Seventh Scanning Electron Microscopy Symposium*, 541-548 (Illinois Institute of Technology Research Institute, 1974).
⁷ Linthicum, D. S., Sell, S., Wagner, R. M., and Trefits, P., *Nature*, **252**, 173-175 (1974).

Immunogenicity of mouse trophoblast and embryonic sac

ALTHOUGH it is generally accepted that the survival of the mammalian embryo as an intrauterine allograft can in part be attributed to the antigenic neutrality of the trophoblastic barrier of the placenta, the way in which this is achieved is unknown. It has been proposed that the trophoblast may either have an intrinsic deficit of transplantation antigens¹ or that these are present but masked in some way, possibly by a mucosubstance on the cell surface². Apparently convincing evidence for this latter hypothesis came from experiments by Currie *et al.*³ who reported that incubation with the enzyme neuraminidase rendered mouse trophoblast immunogenic on injection into recipient mice. As neuraminidase cleaves terminal 'sialic' (*n*-acetyl neuraminic) acid from underlying amino sugars⁴, it was suggested that antigens were masked by sialomucin on the trophoblast surface. These findings have, however, been disputed⁵ and the issue is unresolved. We now report the results of a collaborative study, using two different experimental approaches, which demonstrates that mouse trophoblast, at least at the early proliferative stage, does not possess any detectable quantity of immunogen masked by a neuraminidase-sensitive cell coat. In contrast, embryonic tissue from the same conceptus is highly immunogenic in similar test situations.

In the first series, groups of C57BL (H-2^b) mice were challenged with A(H-2^a) strain embryonic tissues and their immunogenicity assessed using a modification of a cell-mediated microcytotoxicity assay⁶. At 7.5 d *post coitum* A strain ectoplacental cone (EPC) trophoblast was carefully dissected from its embryonic sac and incubated with *Vibrio cholerae* neuraminidase (Behringwerke) at 250 U ml⁻¹ for 1 h at 37 °C. C57BL mice received five successive grafts of five to eight treated EPC to sites beneath the capsule of kidneys and testes. Each C57BL recipient received a total of not less than 28 EPC. Control C57BL mice were challenged with similar serial transfers of 7.5 d. A strain untreated EPC or embryonic sacs from the same conceptus. Twelve days after the last transfer, the mice were killed, their testes and kidneys examined for evidence of graft proliferation and their spleens made into a cell suspension for testing in the cell-mediated microcytotoxicity assay at spleen cell-target cell ratios of 500:1 and 100:1.

Spleen cells from mice challenged by serial transfer of both untreated EPC and neuraminidase-treated EPC were incapable of killing target embryonic fibroblasts, whereas

Table 2 Effect of neuraminidase on the immunogenicity of mouse EPC and embryonic sac: survival of (C3H×C57BR) F₁ tail skin grafts on pre-challenged mice

C3H recipient treatment	No. of recipients	Mean skin graft rejection time (d)±s.d.
None	6	*13.7±1.21
(C3H×C57Br) F ₁ skin graft	8	7.3±0.5
C3H×C3H embryonic sac	8	12.6±1.5
C3H×C3H EPC	5	13.0±1.9
(C3H×C57BR) F ₁ embryonic sac	7	* 8.7±1.7
(C3H×C57BR) F ₁ EPC	8	12.7±1.3

**P* = 0.001.

those from mice challenged with embryonic sac showed a significant degree of killing at identical spleen cell levels (Table 1). A differential immunogenicity of the two components of the mouse conceptus is therefore detectable using the *in vitro* cell-mediated cytotoxicity assay, and pre-treatment of EPC with neuraminidase does not expose any masked antigens. Histological examination of the final site of EPC transfer revealed the presence of trophoblastic giant cells in a haemorrhagic nodule even when neuraminidase incubation had been used. The dosage of enzyme used (250 U ml⁻¹) is thus not detrimental to trophoblast viability.

In a second series, C3H/He-mg strain male mice were challenged with the F₁ embryonic tissues from C3H/He-mg female×C57BR/cd male matings, and the immunogenicity of the tissues was then assessed by a test skin graft. These strains differ at multiple strong non-H-2 loci, but are both H-2^k. (C3H×C57BR) F₁ EPC or embryonic sacs were incubated separately with *Vibrio cholerae* neuraminidase (Behringwerke 100 U ml⁻¹) for 0.75-1 h at 37 °C, washed extensively and injected intraperitoneally into C3H strain recipients. Each recipient received six embryonic sacs or EPC in phosphate-buffered saline (PBS), a repetition of the schedule used previously^{3,4}. Cell viability after incubation was 75-80% on quadruplicate samplings. Control groups of C3H mice were challenged either by (C3H×C57BR) F₁ skin grafting, by intraperitoneal injections of either C3H neuraminidase-treated EPC or C3H neuraminidase-treated embryonic sacs, or had no treatment. After 7-8 d the C3H mice received a (C3H×C57BR) F₁ tail skin graft, and the rate of skin graft rejection was assessed (Table 2). Challenge with neuraminidase-treated (C3H×C57BR) F₁ cones resulted in a skin grafting rejection time of 12.7 d, which is not significantly different from that of control mice challenged with syngeneic embryonic tissues nor from the response to a first set (C3H×C57BR) F₁ skin graft, whereas challenge with neuraminidase-treated (C3H×C57BR) F₁ embryonic sacs accelerated skin graft rejection to a level comparable with that of a second-set reaction (*P*=0.001).

Neuraminidase-treated EPC, as well as untreated trophoblast, thus failed to immunise effectively a recipient when tested either by skin grafting or by cell-mediated lysis of target cells *in vitro*. In contrast, the other component of the post-implantation conceptus, the embryonic sac, was clearly immunogenic in these conditions. This implies that the sac possesses those determinants involved in the stimulation of lymphocyte proliferation as well as transplantation alloantigens, whereas trophoblast must be deficient in at least one of these. Other recent studies have shown that there are no detectable transplantation antigens on mouse EPC trophoblast, as demonstrated by its insusceptibility in an *in vitro* cell-mediated cytotoxicity test⁷, which requires only alloantigenic differences.

The failure to unmask transplantation antigens on neuraminidase-treated EPC is in accord with the report from Simmons' laboratory⁸, and it is difficult to reconcile these findings with the earlier claim of Currie *et al.* It is highly improbable that masking of trophoblast transplanta-

Table 1 Effect of spleen cells from C57BL mice challenged with A strain embryonic sac or EPC tissue on A strain fibroblasts *in vitro*

Immunisation	Cytotoxic effect*
None	—
Ectopic EPC	—
Ectopic neuraminidase EPC	—
Ectopic sac	+++

*Cytotoxic effect scored as +++ total destruction, ++ extensive, + slight, as compared with control cultures, scored —. Scoring based on the assessment of 12 replicate wells in each of three separate experiments. Fibroblasts seeded at 10³ or 10⁴ cells per well in Cooke microtitre plates in medium RPMI +10% FCS, and allowed to plate out for 24 h before the addition of 5×10⁵, or 10⁶, spleen cells, respectively. Cultures were fixed, stained and assessed after a further 48 h.

tion antigens is a strain-specific phenomenon and higher levels of neuraminidase than those used in the present investigations are detrimental to trophoblast cell viability^{5,7}. The effectiveness of the enzyme treatment in removing cell-surface sialomucin was also confirmed by electron histochemical methods carried out in parallel on EPC outgrowths *in vitro*. The degree of colloidal iron binding to trophoblast cell-surface mucoprotein was significantly reduced after neuraminidase incubation. A number of possible explanations have been advanced to account for the differing reports^{7,8}, including contamination of the EPC inoculum by embryonic sac material, which has been demonstrated to be rendered even more immunogenic after enzyme treatment⁹. There is also the possibility of contamination by maternal cells, which was avoided in the present study by the use of F₁ trophoblast.

The finding of a differential immunogenic status of mouse EPC and embryonic sac may be related to their early differentiation⁸. Only weak transplantation antigens, the non-H-2 histocompatibility antigens, have so far been detected on the preimplantation mouse blastocyst⁹, and it has been suggested that the antigenic component may in fact be the inner cell mass or its derivatives rather than the outer trophoblast layer, which gives rise to trophoblast^{10,11}. This is supported by the finding that antigens are not detectable on trophoblast cells after attachment and outgrowth of the blastocyst *in vitro* either by a cell-mediated cytotoxicity test⁷, indirect immunofluorescence (ref. 12 and M.H.J., unpublished) or peroxidase-labelled alloantisera binding (R.F.S., and E.J.J., unpublished).

It can be concluded that early proliferating EPC trophoblast does not possess detectable transplantation antigens. This may not necessarily be the case for differentiated trophoblast of the mature placenta, although the presence of such antigens has not been unequivocally demonstrated as yet. The possible masking effect of overlying membrane-bound mucoproteins at this later stage must also be considered¹³.

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Specific roles for platelet surface glycoproteins in platelet function

THE haemostatic role of the blood platelet is largely dependent on its ability to adhere to exposed subendothelial components in the event of vessel damage¹ and respond to specific aggregation-inducing stimuli, such as adenosine diphosphate (ADP), which seem to act at defined receptor sites on the platelet membrane². The early stages of platelet adhesion and aggregation are surface-mediated phenomena and much interest has centred on determining which of the surface groupings are involved in these mechanisms. Iodination techniques^{3,4} have revealed that a limited number of proteins are exposed on the surface of the platelet and that among these are the major membrane glycoproteins. A possible involvement of carbohydrate groupings in the mechanisms of aggregation as induced by ADP and 5-hydroxytryptamine was described by Mester *et al.*⁵ who showed that the velocity of aggregation was altered by changes in the sialic acid content of the platelet.

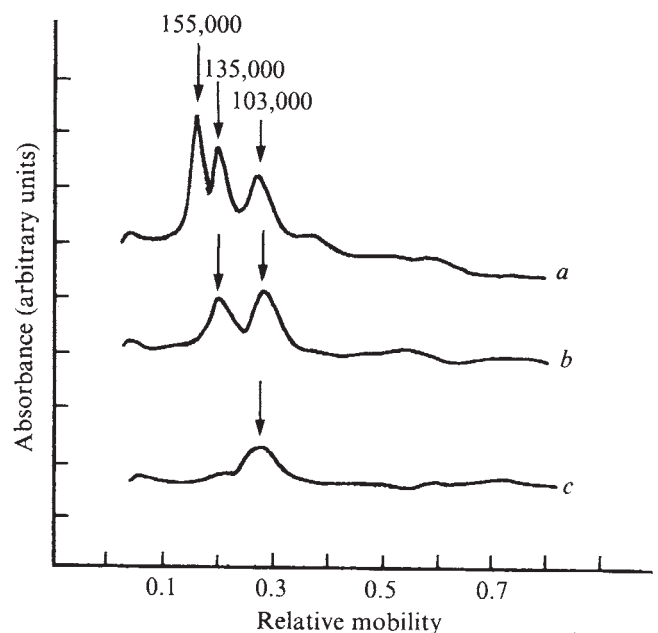


Fig. 1 Polyacrylamide gel electrophoresis in the presence of SDS showing the platelet membrane glycoprotein distribution after the digestion of washed human platelets with trypsin for 0 min (a); 3 min (b); and 15 min (c) before lysis and membrane fractionation. Washed human platelets suspended at $2 \times 10^9 \text{ ml}^{-1}$ in 0.01 M Tris HCl, pH 7.4, containing 0.15 M NaCl and 3 mM EDTA were incubated at 37 °C for 2 min followed by the addition of trypsin (0.2 mg ml^{-1} , Type I Sigma). At selected time intervals aliquots were withdrawn and added to soybean trypsin inhibitor (0.4 mg ml^{-1} , Type I-S Sigma). The platelets were sedimented at 2,000g at 4 °C and the supernatant removed. The trypsin-treated platelets were resuspended at $2 \times 10^9 \text{ ml}^{-1}$ in the above buffer and platelet lysis by freeze-thaw and sonication was performed as described previously⁷. Intact platelets and large particles were removed by centrifugation at 2,000g for 15 min at 4 °C. The supernatant in 2 ml aliquots was centrifuged at 65,000g for 1 h at 4 °C over a 0.5 ml 30% w/v sucrose cushion. The membrane band at the interface was washed three times and resuspended at a protein concentration of 3 mg ml^{-1} . The membranes were solubilised at 37 °C for 2 h following the addition of 2% w/v SDS and 2% v/v 2-mercaptoethanol. Overnight dialysis against 0.01 M phosphate buffer pH 7.1, containing 0.1% w/v SDS and 0.1% v/v 2-mercaptoethanol and electrophoresis on 7% w/v polyacrylamide gels in the presence of 0.02% w/v SDS was performed according to Weber and Osborn¹⁸, 150 μg of solubilised membrane protein being applied to each gel. Glycoprotein was located by the periodic acid-Schiff reaction²⁰ and densitometric scanning was performed using an ISCO model UA4 absorbance monitor. The apparent molecular weights of the glycoproteins were calculated relative to the migration⁷ of a series of protein standards and are shown in the figure.

¹ Simmons, R. L., Cruse, V., and McKay, D. G., *Am. J. Obstet. Gynec.*, **97**, 218–230 (1967).

² Kirby, D. R. S., Billington, W. D., Bradbury, S., and Goldstein, D. J., *Nature*, **204**, 548–549 (1964).

³ Currie, G. A., van Doorninck, W., and Bagshawe, K. D., *Nature*, **219**, 191–192 (1968).

⁴ Gottschalk, A., *Biochim. biophys. Acta*, **23**, 645–646 (1957).

⁵ Simmons, R. L., Lipschultz, M. L., Rios, A., and Ray, P. K., *Nature new Biol.*, **231**, 111–112 (1971).

⁶ Takasugi, M., and Klein, E., *Transplantation*, **9**, 219–227 (1970).

⁷ Jenkinson, E. J., and Billington, W. D., *Transplantation*, **18**, 286–289 (1974).

⁸ Johnson, M. H., in *Clinical and Experimental Immunoreproduction*, 1 (edit. by Edwards, R. G., Howe, C. W. S., and Johnson, M. H.), 87–112 (Cambridge University Press, 1975).

⁹ Searle, R. F., Johnson, M. H., Billington, W. D., Elson, J., and Clutterbuck-Jackson, S., *Transplantation*, **18**, 136–141 (1974).

¹⁰ Billington, W. D., in *Immunology of Reproduction* (edit. by Bratanov, K.), 475–479 (Bulgarian Academy of Science Press, Sofia, Bulgaria, 1973).

¹¹ Gardner, R. L., Johnson, M. H., and Edwards, R. G., in *Immunology of Reproduction* (edit. by Bratanov, K.), 480–485 (Bulgarian Academy of Science Press, Sofia, Bulgaria, 1973).

¹² Heyner, S., *Transplantation*, **16**, 675–678 (1973).

¹³ Billington, W. D., in *Clinical and Experimental Immunoreproduction*, 1 (edit. by Edwards, R. G., Howe, C. W. S., and Johnson, M. H.), 67–85 (Cambridge University Press, 1975).

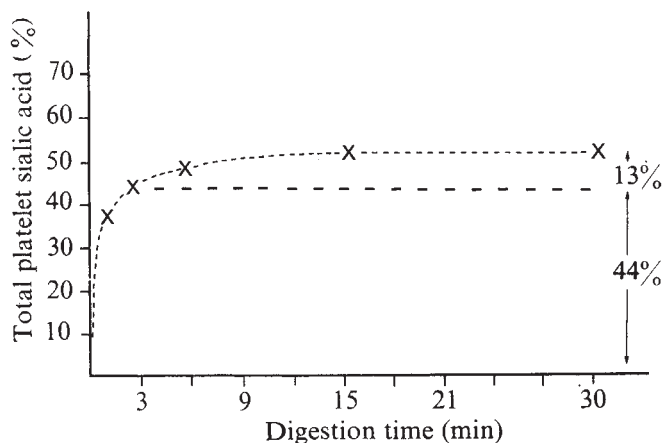


Fig. 2 The rate of release of sialic acid from washed control human platelets by trypsin. Aliquots (0.5 ml) of the supernatants obtained following the trypsin digestion of washed human platelet suspensions as described in Fig. 1 were hydrolysed for 1 h at 80 °C with 0.1 N H_2SO_4 and the sialic acid estimated by the method of Aminoff²¹. The percentage of the total platelet sialic acid (estimated as described previously⁷) released concomitant with the disappearance of the 155,000 molecular weight glycoprotein after 3 min digestion, and the disappearance of the 135,000 molecular weight glycoprotein after 15 min digestion (as shown in Fig. 1) is given.

Here we describe how the bulk of the platelet sialic acid is associated with the major platelet glycoprotein (molecular weight 155,000) and how there are specific defects in the membrane glycoproteins of platelets obtained from patients with clinically defined bleeding disorders as a result of defects in different aspects of platelet function. Human platelet membranes contain three major glycoproteins^{6,7} as characterised by their rate of migration on polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS). Attempts at purifying these glycoproteins to establish their identity have been hindered by their tendency to associate following their solubilisation from the membrane⁸. Figure 1, however, shows how the membrane glycoproteins are differentially degraded during the incubation of washed suspensions of human platelets with trypsin. By calculating the amount of sialic acid released concomitant with the loss of the staining capacity of each of the glycoproteins it was shown that the 155,000 molecular weight glycoprotein contained the predominant part of the trypsin-releasable sialic acid (Fig. 2). Approximately 30 min digestion were required to remove all of the membrane glycoproteins as located by the periodic acid-Schiff reaction, and during this time the bulk of the platelet protein remained resistant to proteolytic attack. The yield of membrane protein obtained from washed platelets digested for 30 min with trypsin (5–7% of the total platelet protein) was not significantly different from that obtained from untreated platelets. Thrombin, when added to washed platelet suspensions, attacks only a limited number of sites of the intermediately migrating major membrane glycoprotein⁹, and releases only 12% of the total platelet sialic acid¹⁰.

In view of the high sialic acid content of the 155,000 molecular weight glycoprotein it would seem that this must be a much branched structure and it is probable that the mutual repulsion of the negatively charged sialic acid moieties, which only occur as the terminating monosaccharide of the glycoprotein oligosaccharide chains, gives the 155,000 molecular weight glycoprotein an extended three-dimensional configuration different from the 135,000 and the 103,000 molecular weight glycoproteins. We believe it significant, therefore, that we have been unable to locate more than traces of the 155,000 molecular weight glycoprotein in membrane fractions prepared from the platelets of two patients with the Bernard-Soulier syndrome (Fig. 3). This is a bleeding disorder associated with an impaired platelet adhesion to subendothelium¹¹ in spite of an apparently

normal mechanism of ADP-mediated aggregation¹². The glycoprotein abnormality is consistent with a previous report which described a reduced sialic acid content and a reduced electrophoretic mobility of the Bernard-Soulier platelets¹³. SDS electrophoresis of solubilised whole platelets confirmed the much reduced content of the 155,000 molecular weight glycoprotein and failed to locate any further gross abnormalities in the other major glycoprotein and protein constituents. It seems to us that as a result of the probable exposed position of the 155,000 molecular weight glycoprotein that it is in a good position to act as the acceptor/receptor on the platelet surface for the macromolecular elements of the subendothelium, adhesion to which is impaired in the Bernard-Soulier syndrome¹¹.

This is in contrast to the apparent absence of the 135,000 molecular weight glycoprotein which we have described previously⁷ in the platelets of three patients with Glanzmann's thrombasthenia, a bleeding disorder associated solely with a defective mechanism of ADP-mediated aggregation¹⁴. The abnormality has now been confirmed in the membranes of the platelets of a further two patients with thrombasthenia (Fig. 3), and would seem not to be associated with the first stage of the ADP aggregation mechanism as the thrombasthenic platelets respond to the initial ADP stimulus with a quantitatively normal shape change (A.T.N. and J.P.C., unpublished), a morphological shape change being the first response of the platelet to stimulation¹⁵. It would seem that the glycoprotein abnormality in the thrombasthenic platelets may well be manifested at a later, possibly enzymatic, stage in the aggregation mechanism. Note that Evans¹⁶ has described a glycoprotein of a similar size, which is an enzyme associated with nucleotide metabolism in the membranes of hepatocyte cells.

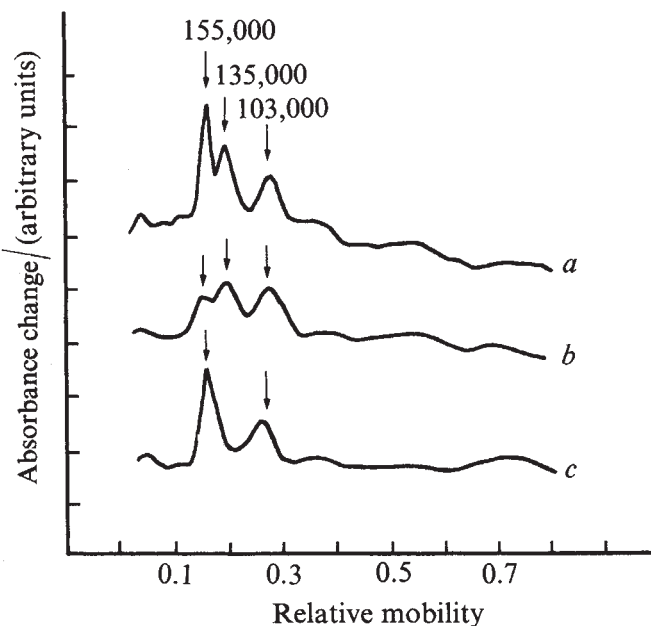


Fig. 3 Polyacrylamide gel electrophoresis in the presence of SDS showing the distribution of glycoprotein in membranes obtained from control human platelets (a); Bernard-Soulier platelets (b) and thrombasthenic platelets (c). Experimental procedures are as described in the legend to Fig. 1. Solubilised membrane protein (150 μ g) was applied to each gel.

It would seem therefore that the layer of bound carbohydrate first shown to be present at the surface of the platelet by electron microscopic techniques¹⁷ contains groupings which are vital to the haemostatic function of the platelet. It is interesting that differences in the glycoprotein composition of the membranes of two strains of amoebae, one of which clumps during exponential growth, have recently been described¹⁸. Evidence for a general role for specific membrane glycoproteins

in the mechanism of cell adhesion and possibly aggregation seems to be accumulating.

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- ¹ Baumgartner, H. R., *Thromb. Diath. Haemorrh. Supp.*, **11**, 161–176 (1972).
- ² Born, G. V. R., in *Human Blood Coagulation, Haemostasis and Thrombosis* (edit. by Biggs, R.), 159–175 (Blackwell, Oxford, 1972).
- ³ Phillips, D. R., *Biochemistry*, **11**, 4582–4588 (1972).
- ⁴ Tanner, M. J. A., Boxer, D. H., Cumming, J., and Verrier-Jones, J., *Biochem. J.*, **141**, 909–911 (1974).
- ⁵ Mester, L., Szabados, L., Born, G. V. R., and Michal, F., *Nature new Biol.*, **236**, 213–214 (1972).
- ⁶ Nachman, R. L., and Ferris, B., *J. biol. Chem.*, **247**, 4468–4475 (1972).
- ⁷ Nurdén, A. T., and Caen, J. P., *Br. J. Haemat.*, **28**, 253–260 (1974).
- ⁸ Lombart, C., Okumura, T., and Jamieson, G. A., *FEBS Lett.*, **41**, 30–34 (1974).
- ⁹ Phillips, D. R., and Poh Agin, P., *Biochim. biophys. Acta*, **352**, 218–227 (1974).
- ¹⁰ Hagen, L., *Biochim. biophys. Acta*, **273**, 141–148 (1972).
- ¹¹ Weiss, H. J., et al., *Am. J. Med.*, **57**, 920–931 (1974).
- ¹² Howard, M. A., Hutton, R. A., and Hardisty, R. M., *Br. Med. J.*, **2**, 586–588 (1973).
- ¹³ Grottum, K. A., and Solum, N. O., *Br. J. Haemat.*, **16**, 275–290 (1969).
- ¹⁴ Hardisty, R. M., in *Human Blood Coagulation, Haemostasis and Thrombosis* (edit. by Biggs, R.), 175–187 (Blackwell, Oxford, 1972).
- ¹⁵ Caen, J. P., and Michel, H., *Nature*, **240**, 148–149 (1972).
- ¹⁶ Evans, W. H., *Nature*, **250**, 391–394 (1974).
- ¹⁷ Behnke, O., *J. Ultrastruct. Res.*, **24**, 51–69 (1968).
- ¹⁸ Hoover, R. L., *Expl Cell Res.*, **87**, 265–276 (1974).
- ¹⁹ Weber, K., and Osborn, M., *J. biol. Chem.*, **244**, 4406–4412 (1969).
- ²⁰ Zacharius, R. M., Zeli, T. E., Morrison, J. H., and Woodlock, J. J., *Analyt. Biochem.*, **30**, 148–152 (1969).
- ²¹ Aminoff, D., *Biochem. J.*, **81**, 384–390 (1961).

Effect of cholesterol on the non-electrolyte permeability of planar lecithin membranes

It is well known that the addition of cholesterol to artificial lipid membranes decreases their permeability. Most of the studies on artificial systems have been performed on liposomes^{1–4}, whereas the effect of the cholesterol on planar lipid bilayers was investigated on water transport only. In particular Finkelstein and Cass⁵ found that addition of cholesterol to membrane of lecithin resulted in a decrease of water permeability. In the present work we studied the effect of cholesterol addition on the non-electrolyte permeability of lecithin planar membranes. The artificial membranes were formed with egg lecithin dissolved in decane to form a 0.5% solution. Lecithin was extracted from egg yolks⁶ and purified by alumina chromatography. Cholesterol, twice crystallised, was added to lecithin solution to obtain cholesterol–lecithin molar ratios of 1:1 or 2:1.

The membranes were formed at 28 °C by brushing the solution on a small circular aperture (0.07 cm²) of a Teflon cell. The cell was similar to that described by Läger *et al.*⁷. The hole of the cell was pre-painted with membrane solution under nitrogen⁸. The bathing fluid was a 10 mM KCl solution. The non-electrolyte permeability was measured as follows. When membrane became “black” about 10 µl of water, containing about 10 µCi of a labelled molecule were added to one side, and the same quantity of unlabelled solution was added to the opposite side. After 40 min a small amount of fluid from both compartments was collected. Stirring of the solutions was achieved by convection currents provided by small temperature differences between the bottom and the top of the chambers. The labelled molecules we used were: ¹⁴C urea, ¹⁴C thiourea, 1-¹⁴C glycerol, N-N'-dimethyl-³⁵S-thiourea (Radiochemical Centre); ¹⁴C acetamide, N-¹⁴C methylurea (New England Nuclear); ¹⁴C formamide, N-dimethyl-carbonyl-¹⁴C formamide (Mallinckrodt Nuclear). The radioactivity readings were performed with a Tri Carb (3320-01) Packard liquid scintillation spectrometer and the permeability coefficients (*P*) calculated.

Table 1 Permeability coefficients (*P*) of non-electrolytes across lipid bilayer membranes

Molecule	Membranes of lecithin in <i>n</i> -decane; $P=10^{-6}$ cm s ⁻¹	Membranes of lecithin cholesterol (1:1) in <i>n</i> -decane; $P=10^{-6}$ cm s ⁻¹
	(Mean ± s.e.)	(Mean ± s.e.)
Glycerol	7.4 ± 0.8 (6)	11.9 ± 2.6 (5)
Urea	3.5 ± 0.5 (12)	3.6 ± 0.5 (13)
N-methylurea	39.2 ± 7.6 (6)	31.4 ± 6.6 (7)
Thiourea	4.3 ± 0.5 (12)	6.3 ± 1.2 (16)
Acetamide	145.1 ± 28.5 (10)	47.8 ± 7.6 (11)
Formamide	146.4 ± 27.8 (11)	67.4 ± 14.0 (6)
N-N'-dimethyl-thiourea	186.7 ± 29.0 (10)	45.5 ± 13.4 (7)
N-dimethyl-formamide	113.4 ± 17.2 (7)	74.7 ± 11.1 (6)

Number of experiments in parentheses.

It can be seen (Table 1) that addition of cholesterol to lecithin membranes (1:1 molar ratio in the membrane solution) does not affect equally the permeability of all the tested molecules. In particular the permeability of the more permeable molecules, that is, formamide, acetamide, N-dimethyl-formamide, N-N'-dimethyl-thiourea is lowered: on the contrary molecules showing a low permeability coefficient through lecithin films that is glycerol, urea and thiourea are completely insensitive to the addition of cholesterol. A similar phenomenon has been observed in biological membranes and proteins have been invoked to explain this selectivity⁹. It is instructive to see, however, that the lipid moiety of simple model membrane may account for this selectivity also.

It is interesting that selectivity phenomena were observed by Cohen and Bangham¹⁰ in the permeation of non-electrolytes through liposome membranes.

The permeability of N-methylurea, showing an intermediate value between the two groups of molecules is scarcely affected by the cholesterol addition. Only an increase of the cholesterol–lecithin ratio in the membrane solution (Table 2) results in a significant decrease of thiourea and methylurea permeabilities.

We can regard the nature of this selectivity displayed by the thin lipid membranes as a process in which at least three rate constants are involved in the permeation of the solutes^{11,12}. Accordingly, in the overall process of permeability we can assume that $K_1 = K_3$ are the rate constants at the interface (water–membrane, membrane–water) and K_2 refers to the hydrocarbon core of the membrane. According to this, K_2 is greater than $K_1 = K_3$ for molecules like formamide, whereas this difference is less marked for urea, glycerol and thiourea. The discrepancy between the effect of cholesterol on the permeability of the two groups of molecules may be explained assuming that cholesterol exerts its condensing effect mainly on the membrane core and its presence scarcely effects the interface. In other words the presence of cholesterol results in a marked decrease of K_2 for molecules like N-N'-dimethyl-thiourea, whereas its effect is relatively small for molecules like urea, whose K_2 value is low. But a further increase of cholesterol content in membrane solution seems to affect the K_2 value of thiourea and N-methylurea.

Evidently the cholesterol concentration in the membrane reaches such a value that affects the membrane structure by making it more compact. In these conditions the membrane permeability approaches that of oxidised cholesterol ones,

Table 2 Permeability coefficients (*P*) of non-electrolytes across lipid bilayer membranes.

Molecule	Membranes of lecithin cholesterol (1:2) in decane $P=10^{-6}$ cm s ⁻¹
	(Mean ± s.e.)
Thiourea	2.4 ± 0.3 (8)
N-methylurea	11.7 ± 3.6 (7)

Number of experiments in parentheses.

which show a marked reduction of permeability as compared with that of lecithin films¹¹.

A different pattern is, however, shown by cholesterol on various artificial membranes. In fact if we compare our results on planar lipid membranes to that obtained on liposomes¹⁻⁴ we observe that the lecithin liposomes are more sensitive to the addition of cholesterol. This different effect of the cholesterol could be due to the presence of decane in lipid films. The solvent molecule could lower the cholesterol-lecithin ratio in the membrane by decreasing the possibility of interaction between these molecules. We have no evidence supporting this idea, but Montal and Mueller¹³ have observed some differences between planar lipid bilayers made with and without hydrocarbon solvent.

On the other hand, Hauser *et al.*¹⁴ have observed that the presence of defected liposomes seriously affects the exact estimation of their permeability. It is possible that cholesterol, by making the liposome structure more compact, reduces the number of defected liposomes, and consequently their permeability is more affected as compared with planar lipid membranes.

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- ¹ De Gier, J., Mandersloot, J. G., and Van Deenen, L. L. M., *Biochim. biophys. Acta*, **150**, 666-675 (1968).
- ² Demel, R. H., Bruckdorfer, K. R., and Van Deenen, L. L. M., *Biochim. biophys. Acta*, **255**, 321-330 (1972).
- ³ De Kruyff, B., Demel, R. H., and Van Deenen, L. L. M., *Biochim. biophys. Acta*, **255**, 331-347 (1972).
- ⁴ Lelievre, J., and Rich, G. T., *Biochim. biophys. Acta*, **298**, 15-26 (1973).
- ⁵ Finkelstein, A., and Cass, A., *Nature*, **216**, 717-718 (1967).
- ⁶ Pangborn, M. C., *J. Biol. Chem.*, **188**, 471-476, (1951).
- ⁷ Laüger, P., Lesslauer, W., Marti, E., and Richter, J., *Biochim. biophys. Acta*, **135**, 20-32 (1967).
- ⁸ Bunce, A. S., and Hider, R. C., *Biochim. biophys. Acta*, **363**, 423-427 (1974).
- ⁹ Handler, J. S., Sugita, M., Preston, A. S., and Orloff, J., *Proc. fourth int. Congr. Nephrol.*, **257** (abstr.) (1969).
- ¹⁰ Cohen, B. E., and Bangham, A. D., *Nature*, **236**, 173-174 (1972).
- ¹¹ Gallucci, E., Micelli, S., and Lippe, C., *Archs int. Physiol. Biochem.*, **79**, 881-887 (1971).
- ¹² Diamond, J., and Katz, Y., *J. Membrane Biol.*, **17**, 121-154 (1974).
- ¹³ Montal, M., and Mueller, P., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 3561-3566 (1972).
- ¹⁴ Hauser, H., Phillips, M. C., and Stubbs, M., *Nature*, **239**, 342-344 (1972).

Sodium transport and lithium movements across the insect blood-brain barrier

INSECTS are the only invertebrate animals which have been shown to possess a well developed blood-brain barrier. This barrier limits the rate of intercellular diffusion of water-soluble substances between the blood and the fluid layer which is the immediate environment of the nerve cells (see ref. 1). It has been proposed that this limitation results from the presence of intercellular occlusions, notably tight junctions, in the cellular layer of the nerve sheath, the perineurium (Fig. 1).

Insects also seem to be unique in their ability to regulate the ionic composition of the nerve cell environment. It has frequently been postulated that this ionic homeostasis results from active transport by the perineurium and underlying glial elements (Fig. 1). In particular, it has been suggested that sodium ions are transported from the blood to the fluid layer at the neuronal surfaces to maintain the action potentials. We present evidence which accords with this hypothesis.

We have used action potential amplitude as an indicator of extra-axonal sodium concentration in isolated central nervous connectives of the cockroach *Periplaneta americana*. The rates of change in concentration were calculated in different experimental conditions with intact preparations and with those in which the perineurium was surgically removed (desheathed).

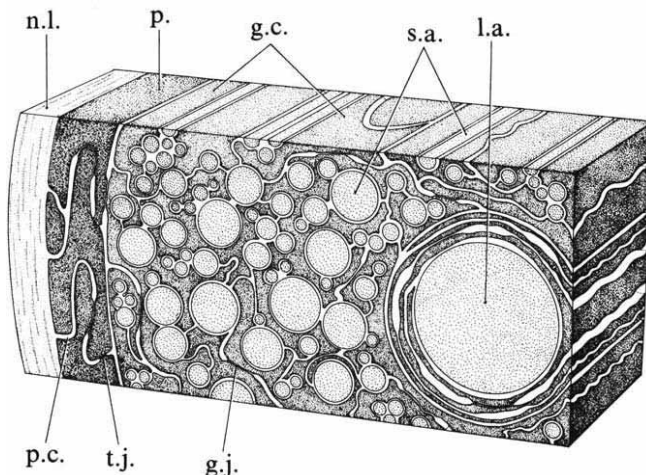


Fig. 1 Schematic view of a portion of the periphery of a cockroach central nervous connective. The superficial connective tissue sheath, the neural lamella, overlies the cellular perineurium. Beneath, the axons are enveloped, to varying degrees, by glial processes. Intercellular movements of ions and molecules through the perineurium are restricted by junctions in the perineurium²⁻⁴. In addition to the tight junctions invariably present at the inner ends of the clefts, there are occasional, more peripherally located, septate desmosomes. Glial elements are linked to one another, and adjacent perineurial cells, by gap junctions. n.l., Neural lamella; p., perineurium; g.c., glial cells; s.a., small axons; l.a., large axon; p.c., perineurial cleft; t.j., tight junction; g.j., gap junction.

Figure 2 shows the effects of exposure of an intact preparation to sodium-deficient (Tris-substituted) Ringer. The decline in amplitude of the action potentials, and the loss of sodium indicated by this, are slow compared with the rapid recoveries obtained on return to normal Ringer. This asymmetry, which is absent in desheathed preparations, accords with the above concept of an inwardly-directed transport of sodium ions. The rates of decline varied in different preparations, whereas the rates of subsequent recovery were remarkably constant. The following experiments describe factors which affect this recovery rate.

In spite of the ability of lithium ions to substitute for those of sodium in axonal conductance in desheathed preparations, results were obtained in which no recovery in the amplitude of the action potentials occurred when sodium-depleted connectives were exposed to Ringer in which sodium was replaced by lithium (Fig. 3). A rapid recovery was observed, however, in lithium Ringer in preparations exposed briefly to hypertonic urea solution, a procedure which is known to by-pass the peripheral blood-brain barrier⁶. The specificity for sodium is unlikely to result from passive permeability properties of the system⁹ and again suggests that there is an inwardly-directed transport of sodium ions to the extra-axonal fluid. The slow recovery of the action potentials on return to normal saline (Fig. 3) could result from the effects of lithium on sodium pumping or (in view of the loss of sodium from connectives being greater in lithium than in Tris-substituted Ringer⁸) from a delay involved in re-establishing the levels of sodium available to a pump.

Externally-applied amiloride (1 mM) and ouabain (10 mM), which is known to affect the axonal sodium pump⁷, were found to be without effect on the rate of recovery of the action potentials in intact, sodium-depleted connectives. The rate of recovery was, however, reduced in the presence of 2,4-dinitrophenol, which at 50 μ M did not affect axonal responses in desheathed preparations. In spite of its lack of effect on the axonal sodium pump⁷ the sodium-transport inhibitor, ethacrynic acid, reduced the apparent movement of sodium ions to the axonal surfaces (Fig. 4). It can be seen that this inhibitor

also seemed to reduce the outward movement of sodium ions.

These effects of ethacrynic acid accord with previous observations on cockroach connectives which showed that, although the axonal sodium pump is insensitive, extra-axonal elements are affected by this compound⁷. The lack of effect of ouabain on sodium movements between the external medium and the extra-axonal fluid accords with the apparent insensitivity of the glia to cardiac glycosides as compared with the axons whose sodium pump has been shown to be inhibited by strophanthidin⁷.

A previous study has shown that lithium accumulates in intact connectives to levels similar to those of sodium, and it was suggested that this accumulation occurred in the glial and perineurial elements⁸ which are linked by gap junctions (Fig. 1). This suggestion is further supported by the apparent inability, reported here, of lithium to gain access to the axonal surfaces. In the presence of effective intercellular occlusions in the perineurium, we must assume that lithium can cross the outwardly-facing perineurial membranes. Since the latter have a relatively low passive permeability to lithium and sodium³, these cations are unlikely to enter the perineurial cells by passive diffusion down an electrochemical gradient. The

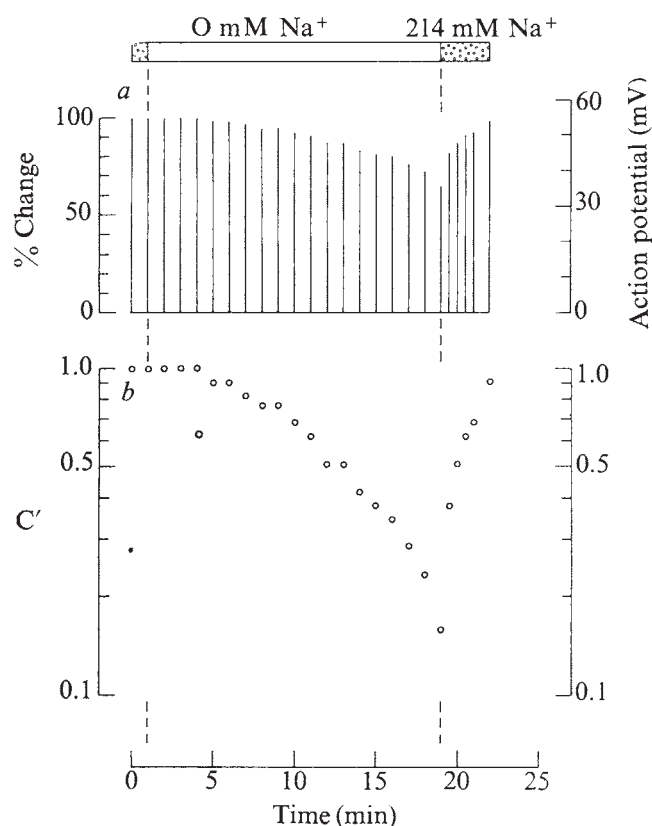


Fig. 2 *a*, Effect of sodium-deficient (Tris-substituted) Ringer on action potential amplitude. In this and subsequent figures, the amplitude of compound action potentials is shown, as recorded with the 'sucrose-gap' technique in cockroach central nervous connective, following electrical stimulation at regular intervals. Nerve cords were isolated and maintained in the high-sodium Ringer of Yamasaki and Narahashi⁵ until a constant action potential amplitude was obtained before the experiments were begun. *b*, Change in relative sodium concentration at the axonal surfaces (C') estimated from percentage change in action potential amplitude shown in (*a*). Initial extra-axonal sodium concentration is unknown and has been designated as 1.0. The change in sodium concentration relative to this value was estimated from the Nernst relationship between sodium concentration and the percentage change in action potential amplitude of surgically-exposed axons in desheathed preparations.

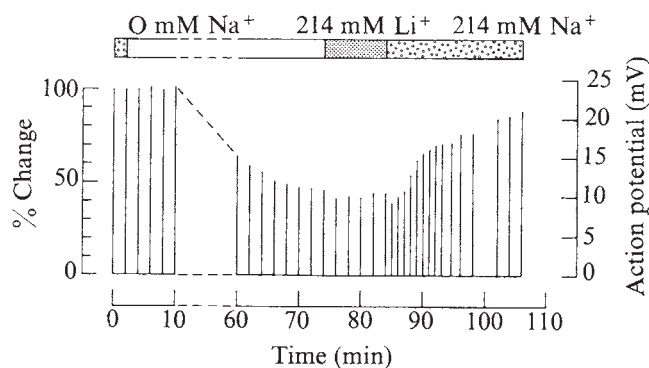


Fig. 3 Effect of exposure to lithium Ringer, following sodium depletion in the sodium-deficient (Tris) Ringer. Subsequent exposure to the original sodium concentration results in a relatively slow recovery of action potential amplitude.

inability of amiloride to block the entry of sodium, and the previous observation of a retention of lithium ions during prolonged washing in normal Ringer⁸, suggest that these cations could share a peripheral, unidirectional, transport system. The exclusion of lithium ions from the fluid bathing the axon surfaces would then be the result of their inability to cross the inwardly-directed perineurial and glial membranes. This inability contrasts with the ready access of sodium ions to the axon surfaces (Fig. 2) and suggests that sodium is transported across the glial membranes by a conventional pump which, as in crustacean neurones⁹, will not accept lithium ions. Such a pump in this position could also support axonal function by transporting potassium ions away from the extra-axonal fluid.

The net inward movements of sodium ions between the external medium and the fluid bathing the axon surfaces therefore seem to be largely mediated by active transport through the perineurial and glial membranes—being inhibited by dinitrophenol and ethacrynic acid. Transport across the

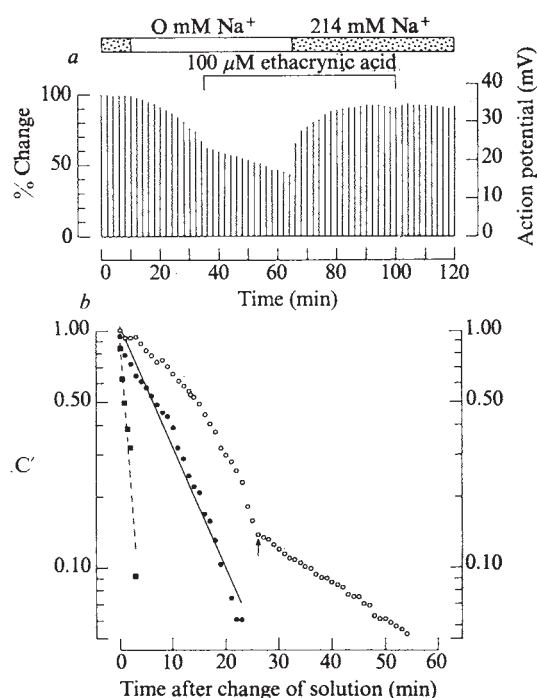


Fig. 4 *a*, Effect of 100 μ M ethacrynic acid on the change in action potential amplitude during and after exposure to sodium-deficient (Tris) Ringer. *b*, Change in relative extra-axonal sodium concentration (C') estimated from (*a*), as in Fig. 2*b*, showing a slowing of the rate of decline (○) following addition of ethacrynic acid (arrow). Recovery rate, plotted as $1 - C'$, in the presence of ethacrynic acid (●) is slower than that observed in an untreated preparation (■). The latter data are taken from the experiment illustrated in Fig. 2.

outer perineurial membranes seems to be effected by a pump which also accepts lithium, and across the inwardly-directed membranes by one which is specific for sodium ions.

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- ¹ Treherne, J. E., in *Insect Neurobiology* (edit. by Treherne, J. E.), 187–244 (North Holland, Amsterdam, 1974).
- ² Treherne, J. E., Lane, N. J., Moreton, R. B., and Pichon, Y., *J. exp. Biol.*, **53**, 109–136 (1970).
- ³ Pichon, Y., Moreton, R. B., and Treherne, J. E., *J. exp. Biol.*, **54**, 757–777 (1971).
- ⁴ Lane, N. J., and Treherne, J. E., *Tissue & Cell*, **4**, 427–436 (1973).
- ⁵ Yamasaki, T., and Narahashi, T., *J. Insect Physiol.*, **3**, 146–158 (1959).
- ⁶ Treherne, J. E., Schofield, P. K., and Lane, N. J., *J. exp. Biol.*, **59**, 711–723 (1973).
- ⁷ Pichon, Y., and Treherne, J. E., *J. exp. Biol.*, **61**, 203–218 (1974).
- ⁸ Bennett, R. R., Buchan, P. B., and Treherne, J. E., *J. exp. Biol.*, **62**, 231–241 (1975).
- ⁹ Baker, P. F., *J. Physiol., Lond.*, **180**, 383–423 (1965).

Limit of fish swimming speed

OBSERVATION and analysis of the swimming of marine fish in laboratory aquaria and in the vicinity of fishing gear (C.S.W., unpublished) have indicated that small fish (0.1 m) can reach speeds up to 25 body lengths s^{-1} whereas larger fish (1 m) seem unable to exceed 4 body lengths s^{-1} . Bainbridge¹ showed that smaller fish were capable of higher tail beat frequencies than larger fish and forward motion resulting from one complete tail beat cycle (or one 'stride') was between 0.6 and 0.8 times the length of the fish. In the present study the tail beat frequency or frequency of strides is considered to be limited by the contraction time of the swimming muscle of the fish, and maximum observed swimming speeds are found to fit the dimensions of the muscle twitch time and stride length.

The twitch contraction time of white lateral muscle (taken from freshly killed marine teleosts, previously adapted to and rested in marine aquaria) was measured by electrically stimulating pieces of their muscle in a temperature-controlled isosmotic bath.

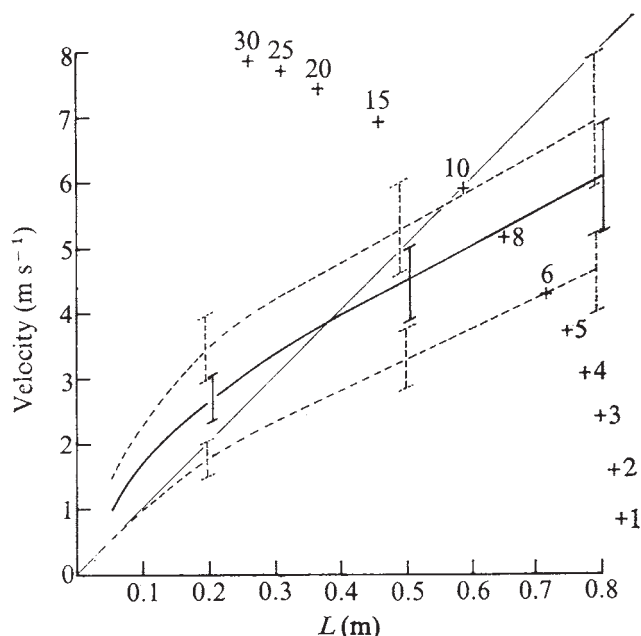
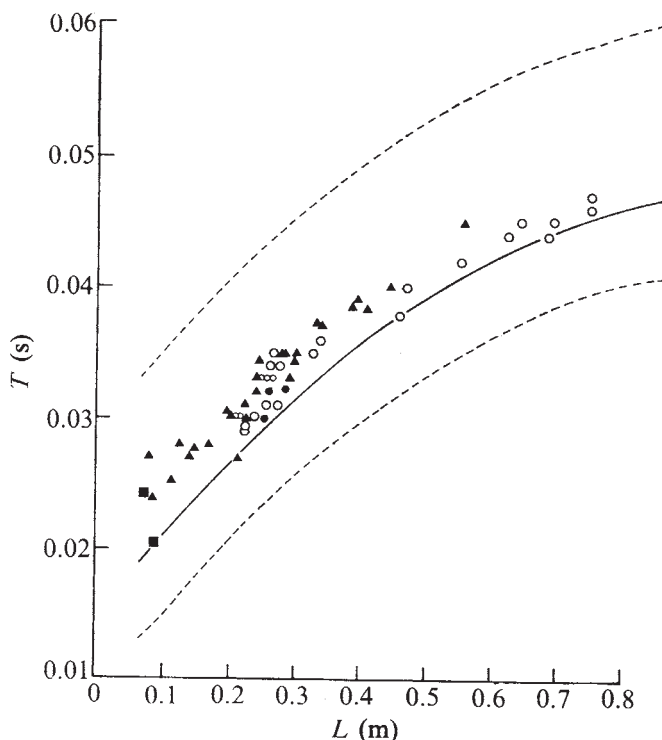


Fig. 2 Maximum swimming speeds predicted by the three muscle contraction time curves in Fig. 1 for a stride length of 0.7 times L ●: top, 20 °C; middle, 14 °C; bottom, 0 °C. Increase in speed as a result of altering A between 0.6 and 0.8 is indicated by the vertical bars. Third curved scale allows conversion of any point on the graph to body lengths s^{-1} by lining up that point with the origin of the graph. A 10 body length s^{-1} line is drawn in.

The time from the stimulating pulse to the peak of contraction was measured for a number of species of marine fish of a wide range of sizes (Fig. 1). From these individual points a continuous line representing the shortest contraction time for each size of fish was drawn. It was then assumed that the shortest time for a complete tail beat cycle or stride was limited by the muscle twitch contraction time of the muscle; for example, a contraction time of 0.04 s for muscle from a fish 53 cm long (Fig. 1) would allow 25 contractions, alternated between the two sets of opposing lateral muscles, per s and thus 12.5 complete tail beats or strides per s. With a maximum stride length of 0.8 times body length the maximum speed possible for the 53-cm fish will be $12.5 \times 0.8 = 10$ body lengths s^{-1} or 5.3 m s^{-1} . In this way the maximum swimming speed M at a stated temperature is predicted by the formula $M = AL/2T$ where L is the length of the fish (m), T is the muscle contraction time (s) and A is the stride length which according to existing measurements lies within the range 0.6–0.8 times L . The calculated maximum swimming speeds for fish with $A = 0.6, 0.7$ and 0.8 and for the shortest muscle contraction times associated with the fish lengths indicated in Fig. 1 are shown in Fig. 2.

Fig. 1 Muscle contraction time for different sizes of teleost: ▲, plaice, *Pleuronectes platessa*; ○, cod, *Gadus morhua*; ■, haddock, *Melanogrammus aeglefinus*; ●, salmon, *Salmo salar*. Dashed thin curves 20 °C (bottom) and 0 °C (top) allow for a measured change in contraction time of 0.001 s for 1 °C change in temperature. The muscle block (3 × 1 × 0.5 cm) was rigidly fixed at one end in a jaw of stainless steel gauze (12 wires, 0.27 mm diameter, per cm) and was attached at the other end by a light hook to a 30 mm optical wedge isotonic transducer (Devices 3194), loaded with a light spring to give a tension of 20 g. Single direct current pulses (0.001 s and 20 V) were applied to the saline bath (14 °C, NaCl 0.2 M) by way of stainless steel vertical plates one on either side of the muscle block. The resulting movement of the muscle was displayed on an oscilloscope, photographed and measured. Although temperature was found to have a marked effect on the time of contraction, 'after-loading' the muscle only altered the amplitude of the movement and not the time for complete contraction.

Movements of swimming fish representing a wide range of sizes of marine species were recorded on videotape using a modified closed circuit television system. Fish in conditions as described previously², were either startled into movement or trained to race between two feeding points by association of underwater flashing lights with the appearance of food. Maximum speeds of 26 body lengths per second were recorded for small haddock and sprats (10 cm) which show evidence of the maximum predicted tail sweep time which was close to the television frame time of 0.02 s and a stride approaching $0.9L$; an example is shown in Fig. 3. Similarly small (seawater) salmon (25–28 cm) reached 2.5 m s^{-1} (10 body lengths s^{-1}) and the tail beat took 0.06 s for a complete cycle or 0.03 s for each muscle to contract. The highest speed for cod yet measured is 2.8 m s^{-1} for a 73-cm fish; however, a single tail-beat cycle at this speed took 0.18 s and its stride was $0.68L$. If this cod had used its shortest predicted tail beat of 0.09 s and had maintained the same stride it would have reached 5.5 m s^{-1} . The race track between the flashing lights in the Aberdeen tank is 8 m and it is likely that this large fish weighing 3.78 kg would not achieve its maximum speed in such confined surroundings.

Using the same stride length this cod may from Fig. 2 be expected to reach 6.6 m s^{-1} at 20°C and only 4.3 m s^{-1} at 0°C . It seems that the shorter contraction times predicted here of the muscle of tropical fish above 20°C may help to explain the remarkable records of the barracuda³, *Sphyræna barracuda* (Walbaum), swimming at 12.2 m s^{-1} ($L=1.22 \text{ m}$), the yellowfin tuna⁴, *Thunnus albacares* (Bon-

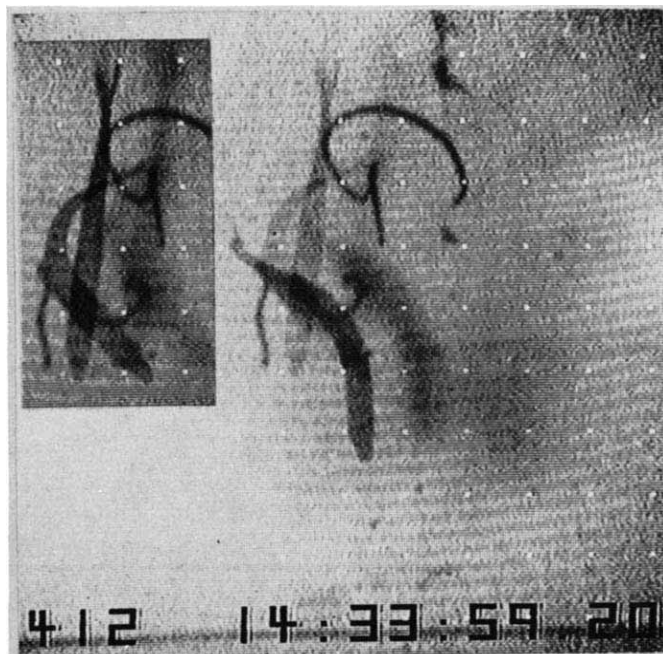


Fig. 4 Two frames 0.02 s apart show three positions of a sprat, *Sprattus sprattus* (L.) (8.3 cm, 12°C). Frame 1 (inset), position 1, time 0.16 s (not indicated) fish is standing still; frame 1, position 2, time 0.18 s tail bent to left; frame 2, position 3, time 0.20 s, tail to right and forward speed is 1.4 m s^{-1} . Note the muscle twitch speed of an 8.3-cm fish is near 0.02 s. Television system as in Fig. 3.

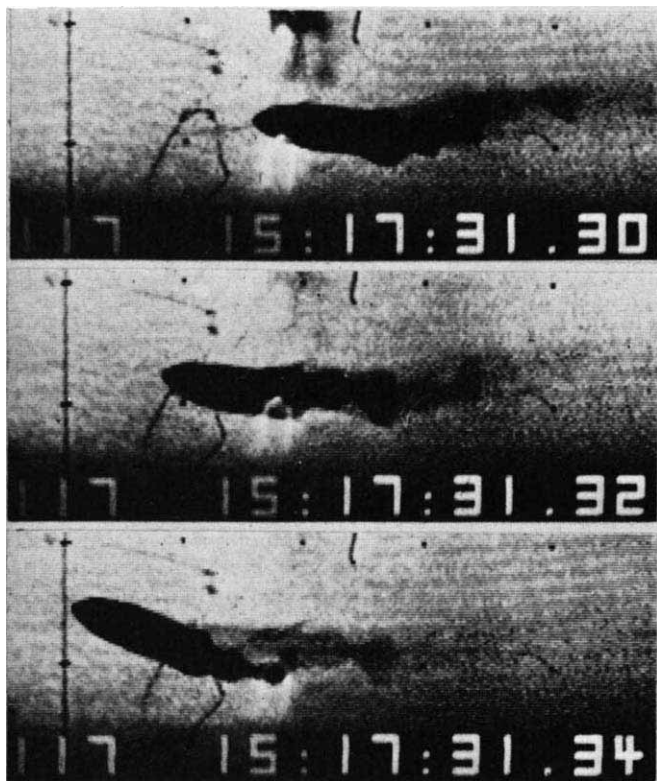


Fig. 3 Startled haddock (10 cm, 12°C) moving at 2.5 ms^{-1} : black dots are 5 cm apart. Three photographs of single television frames are 0.02 s apart. First three digits indicate experiment number, the remaining indicate time. Note the tail is to the right of the fish's body at 0.30, left at 0.32 and right again at 0.34 s. A fish this size has a muscle twitch contraction time of about 0.02 s, approximately matching the television frame rate. Television camera was fitted with a silicon-diode array vidicon tube having high-red sensitivity and short-image retention time. Fish were illuminated with a strobed ($10 \mu\text{s}$ flash), red light source synchronised to the television scan rate and placed next to the camera lens to create frozen silhouettes of moving fish against marked screens of reflex reflector material (3M Scotchlite, 3270).

natare), at 20.8 m s^{-1} ($L=0.98 \text{ m}$) and wahoo⁴, *Acanthocybium solandri* (Cuvier) at 21.4 m s^{-1} ($L=1.13 \text{ m}$), but it will be essential to measure muscle properties at known temperatures and stride lengths for these species if they are to be drawn into the same scheme. It is clear that to make use of the shorter contraction time in the larger fish the muscle must be supported by increased power and as Hill⁵ and Close⁶ have pointed out, in discussing muscle contraction speeds in relation to animal size, the strength of the supporting framework is critically matched to these muscle properties.

It seems that the fish examined here are tailored to supply sufficient power to make full use of their shortest muscle contraction time even when fully loaded. Three frozen images at 0.02 s intervals (Fig. 4), show a sprat (8.3 cm) starting from zero speed which by the third image has travelled forwards at 1.4 m s^{-1} . Each of the opposing muscles even with the load involved in the acceleration have contracted within 0.02 s; note how the loaded single power beat (between positions 2 and 3, Fig. 4) shows a smaller amplitude than the first unloaded positioning bend.

That the initial performance of the fish's rested anaerobic muscle is exceptional, is confirmed by its biochemistry. The glycogen concentration can be as high as 1 g per 100 g muscle: 1 g glycogen releases 558 J when converted to lactic acid. The maximum rate of oxygen uptake in fish is near 1.6 mg oxygen per 100 g per min; limiting aerobic energy release to no more than 36 J per 100 g per min or 0.6 W per 100 g fish weight. Ten per cent of this yield is committed to maintenance⁷. Half of the glycogen fuel store was converted to lactic acid within the first 2 min of severe activity in rainbow trout⁸, *Salmo gairdneri*. Artificial stimulation of loaded isometric plaice muscle (0.001 s, 20 V pulses, 0.02 s apart) has shown complete depletion of glycogen within 30 s. The same pulses applied singly in the same conditions resulted in a 50% drop in the twitch tension after 130 pulses and 80% tension loss after 300 pulses. Glycogen was lost at a similar rate (C.S.W., unpublished). It is clear that the rested fish could use its full contraction

power together with its shortest contraction time to attain maximum speed, but the power to use its shortest contraction time will be lost quickly. These short periods at maximum speed are, however, significant in terms of survival.

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- ¹ Bainbridge, R., *J. exp. Biol.*, **35**, 109–133 (1958).
- ² Wardle, C. S., and Kanwisher, J. W., *Mar. Behav. Physiol.*, **2**, 311–324 (1974).
- ³ Gero, D. R., *Am. Mus. Novit.*, No. 1601, 1–32 (1952).
- ⁴ Walters, V., and Fiersteine, H. L., *Nature*, **202**, 208–209 (1964).
- ⁵ Hill, A. V., *Br. med. Bull.*, **12**, 165–166 (1956); *Sci. Progr.*, **38**, 209–230 (1950).
- ⁶ Close, R. I., *Physiol. Rev.*, **52**, 129–165 (1972).
- ⁷ Brett, J. R., *Resp. Physiol.*, **14**, 151–170 (1972).
- ⁸ Black, E. C., Connor, A. R., Lam, K., and Chiu, W., *J. Fish. Res. Bd Can.*, **19**, 409–436.

Moulting hormone production in the isolated larval abdomen of the Colorado beetle

IN spite of the overwhelming evidence for the essential roles of the prothoracic glands in the synthesis of moulting hormone and the regulation of insect moulting and development^{1–3}, several instances of moulting without prothoracic glands have been reported, either by extirpation (*Galleria mellonella*⁴ and *Periplaneta americana*⁵) or by ligation (*Tenebrio molitor*⁶ and *Dermestes vulpinus*⁷). Little experimental evidence is available to elucidate the significance of this phenomenon. We report here a similar moulting phenomenon observed in the Colorado beetle, *Leptinotarsa decemlineata* (Say), and demonstrate that the isolated abdomen of this species can produce moulting hormone and moult in the absence of the prothoracic glands.

Mature larvae of the Colorado beetle dig into soil and enter the prepupal stage when feeding stops. When these larvae are prevented from digging, pupation is delayed⁸. Before digging occurs, moulting is promoted by ligation between the head and the thorax, even among young larvae^{9,10}. Furthermore, when the ligature is placed between the thorax and the abdomen, the isolated abdomen can moult successively into a pupal and an adult abdomen. The moulting of the isolated abdomen can be obtained in feeding larvae as young as 1.5 d after the last larval moult.

The two most likely hypotheses to explain the nature of this unusual moulting of the isolated abdomen are: sufficient moulting hormone is already present at the time of ligation and persists afterwards; or the moulting hormone is produced by the tissues to induce a moult. We investigated these possibilities by first determining the moulting hormone titres of larval and pupal stages by means of chemical extraction¹¹ and the *Musca* test¹².

The Colorado beetles were reared on potato leaves at 25 °C and a photoperiod of L:D 18:6. In these conditions, the last larval instar requires 8 d (3.5 d feeding stage followed by 4.5 d prepupal stage) and the pupal stage lasts for 6 d. We found no detectable amount of moulting hormone (less than 0.5 *Musca* unit (MU) per gram fresh weight; 1 MU = 3.5 ng of ecdysterone in our test) during the entire last larval feeding stage and the first day of the prepupal stage. Moulting hormone was detected in 2-d-old prepupae and reached a peak of 30 MU g⁻¹ in 3-d-old prepupae. It began to decline in 3.5-d-old prepupae and fell to a low level shortly before the larval–pupal moult. In the pupa, moulting hormone activity began to increase during the first day and reached a peak of 100 MU g⁻¹ on the third day. The titre again declined on the fourth day and became undetectable 1 d before the adult emerged. Our results clearly demonstrate that before or at cessation of feeding, the larva does not contain a detectable amount of moulting hormone in its tissues. Therefore, the

most feasible explanation seems to be the synthesis of moulting hormone by the isolated abdomen.

The ligated abdomen of a mature larva requires about 10 d to moult compared with 4.5 d needed for a normal larva. By injecting graded doses of ecdysterone (0.1–1.0 µg per abdomen), we can gradually shorten the time before moulting, to the normal duration. This result suggests that the delay of moulting in the isolated abdomen may be the result of a slow rate of synthesis or an insufficient amount of moulting hormone in the tissues. We then proceeded to extract moulting hormone from abdomens 1 and 7 d after ligation. We found no detectable amount after 1 d, but obtained an average of 10 MU g⁻¹ of moulting hormone in abdomen 7 d after isolation. The above data prove conclusively that the isolated abdomen of the Colorado beetle is capable of producing sufficient moulting hormone to induce a moult.

The significance of moulting hormone production outside the prothoracic glands is now becoming more evident. Several findings have led us to conclude that in the pupal stage of the Colorado beetle, moulting hormone is produced by the tissues rather than by the prothoracic glands. First, we have observed in this species, as others had reported in the case of *T. molitor*^{13,14}, that the prothoracic glands degenerate soon after the larval–pupal moult, although the highest titre of moulting hormone we detected occurred in the 3-d-old pupa. Second, when newly moulted pupae were ligated between the thorax and the abdomen, we found no difference in the time of the adult moult between the anterior and the posterior portions, indicating that the presence of the neuroendocrine systems is not essential for adult development. Our finding of actual moulting hormone production in the isolated larval abdomen also strongly suggests that a similar mechanism exists in the pupal tissues. This evidence does not exclude the essential role of the prothoracic glands during the larval–larval and larval–pupal moults. By a series of ligation experiments with larvae and prepupae of different age, we established a critical period after which ligature between the thorax and the abdomen no longer influences the timing of the larval–pupal moult, indicating that the prothoracic glands are functional in normal insects. Our results, however, suggest that tissue synthesis of moulting hormone supplements that of the prothoracic glands.

The ability of the isolated larval abdomen to convert isotopically labelled cholesterol into ecdysones has been demonstrated in two species of Lepidoptera, *Bombyx mori*¹⁵ and *Mamestra brassicae*¹⁶. As regards the site of moulting hormone synthesis outside the prothoracic glands, the oenocytes have often been suggested, as in *Calpodex ethlius*¹⁷ and in *T. molitor*¹⁸. Romer *et al.*³ showed that the oenocytes of *T. molitor* larvae can convert ¹⁴C-cholesterol into ecdysterone *in vitro* and concluded that moulting hormone is synthesised by the oenocytes in both larvae and pupae. Whether the oenocytes or other tissues are responsible for the production of moulting hormone in the isolated larval abdomen of the Colorado beetle remains to be demonstrated. The chemical nature of the moulting hormone and the mechanisms by which tissue synthesis is regulated also require further elucidation.

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¹ Chino, H., *et al.*, *Science*, **183**, 529–530 (1974).

² King, D. S., *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 793–796 (1974).

- ³ Romer, F., Emmerich, H., and Nowock, J., *J. Insect Physiol.*, **20**, 1895–2094 (1974).
- ⁴ Piepho, H., *Naturwissenschaften*, **35**, 94–95 (1948).
- ⁵ Chadwick, L. E., *Science*, **121**, 435 (1956).
- ⁶ Stellwaag-Kittler, F., *Biol. Zbl.*, **73**, 12–49 (1954).
- ⁷ Sláma, K., Romanuk, M., and Sorm, F., *Insect Hormones and Bioanalogs* (Springer Wien, New York, 1974).
- ⁸ Hsiao, T. H., and Hsiao, C., *14th Intern. Congr. Ent. Abstr.*, 176 (1972).
- ⁹ Wilde, J. de, *Am. Zool.*, **15** (in the press).
- ¹⁰ Deleurance, S., *C. r. hebd. Séanc. Acad. Sci. Paris D*, **277**, 2177–2180 (1973).
- ¹¹ Karlson, P., and Shaaya, E., *J. Insect Physiol.*, **10**, 797–804 (1964).
- ¹² Staal, G. B., *Proc. K. ned. Akad. Wet. C*, **70**, 409–418 (1967).
- ¹³ Srivastava, U. S., *Experientia*, **16**, 455 (1960).
- ¹⁴ Romer, F., *Z. Zellforsch. mikrosk. Anat.*, **122**, 425–455 (1971).
- ¹⁵ Nakanishi, K., Moriyama, H., Okauchi, T., Fujioka, S., and Koreeda, M., *Science*, **176**, 51–52 (1972).
- ¹⁶ Gersch, M., and Stürzebecher, J., *Experientia*, **27**, 1475–1476 (1971).
- ¹⁷ Bonner-Wier, S., *Nature*, **228**, 580–581 (1970).
- ¹⁸ Romer, F., *Naturwissenschaften*, **58**, 324–325 (1971).

Use of an X-ray television for diffraction of the frog striated muscle

THE exposure time needed to record low angle diffraction patterns from biological materials can be shortened to a large extent by using a sensitive detector instead of X-ray films. One such detector, a position sensitive proportional counter, has proved useful in studying the kinetics of the structural transitions of a lipid membrane¹ and a photo-receptive membrane². We have tried another type of fast detector, an X-ray television, to study the low-angle equatorial diffraction patterns from the frog striated muscle during contraction.

The X-ray television³ consisted of a phosphor screen viewed from behind by an image orthicon tube. The diffraction pattern formed on the phosphor screen was displayed on a TV monitor, and was photographed with high speed film (Kodak Tri-X). The spatial resolution of this detector system was 150 μm on the surface of the phosphor screen.

The sartorius muscle was dissected from a bull frog, *Rana catesbeiana*. The muscle was mounted in a Perspex chamber filled with Ringer's solution (0 °C). The proximal end of the muscle was fixed in the chamber by clamping the pelvic bone with a double hook. The distal end was connected to a tension transducer. The sarcomere length of each specimen was adjusted to 2.2 μm using the diffraction of a laser light ($\lambda = 632.8 \text{ nm}$).

Figure 1a shows a densitometer trace (right half only) of the equatorial pattern of a resting muscle recorded by the TV detector. Two reflections are clearly seen, and are indexed 1,0 and 1,1 from the hexagonal filament lattice⁴. The exposure time needed was 10 s. When X-ray film (Sakura N) was placed in the position of the phosphor screen, an exposure of about 15 min was needed to record the same reflections. Thus the TV detector was about 90 times faster than the film.

The relative strength of the 1,0 and 1,1 reflections can be related to the state of the myosin projections^{5,6}. It is of interest to know whether the TV method provides correct

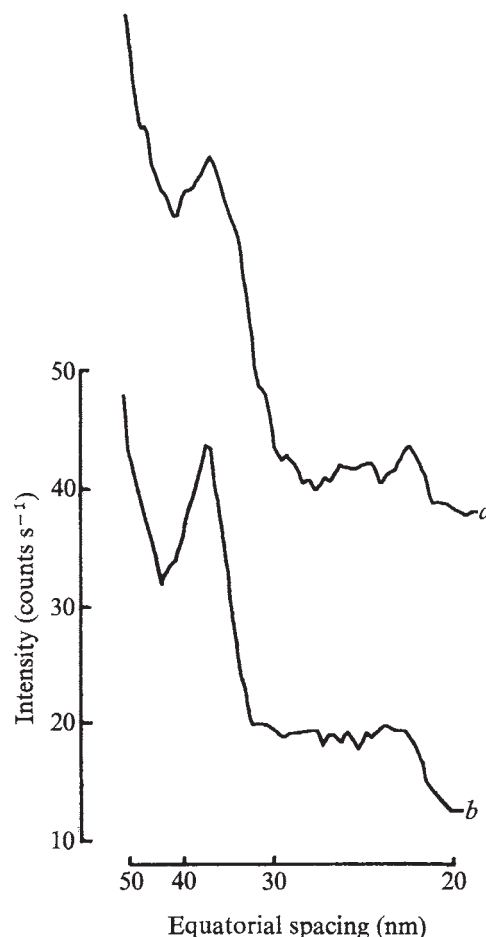


Fig. 1 *a*, A densitometer trace of the equatorial reflections recorded with an X-ray television from a resting frog sartorius muscle at a sarcomere length of 2.2 μm . The muscle was suspended in Ringer's solution (115 mM, NaCl; 2.5 mM, KCl; 1.8 mM, CaCl₂; 66.7 mM, dextrose; 2.15 mM, Na₂HPO₄; 0.85 mM, NaH₂PO₄; pH = 7.2), and was exposed to X rays for 10 s with a specimen-to-detector distance of 75 cm. The X-ray generator was a rotating-anode type (Rigaku RU100) with a line focus (1 × 0.1 mm) on a copper target. This was operated at 40 kV with a tube current of 35 mA. A low-angle camera of Franks type was used to collimate X rays. The TV detector was operated in the integrating scan mode; the scanning beam was switched off during the 10-s exposure so that the diffraction pattern was integrated on the tube target. After the exposure, the integrated pattern was read on several TV frames by switching on the scanning beam. The 1,0 reflection (the peak at the left) was stronger than the 1,1 reflection (the peak at the right). *b*, Intensity distribution of the equatorial reflections obtained from the same specimen using a scintillation counter placed behind a narrow slit (100 μm wide). Each measurement took 100 s, and the slit was moved by 100 μm before each measurement.

information about the intensity ratio ($I_{1,0}/I_{1,1}$). Therefore the intensities of the reflections were measured with a scintillation counter (Fig. 1b), and the ratio obtained was compared with that calculated from the densitometer trace (first row of Table 1). There was no significant difference between the two ratios.

Figure 2a shows a densitometer trace (right half only) of the equatorial reflections of a muscle in rigor. The 1,1 reflection became stronger than that of the resting muscle. Figure 2b shows the intensity measurements from the scintillation counter. The intensity ratio obtained by the TV method corresponded with that obtained from the scintillation counter (second row of Table 1).

When the living muscle was stimulated with supra-maximal pulses, it developed a tension of about 2.4 kg cm⁻². Separate experiments using the light diffraction method showed that the sarcomere length of the muscle, which was fixed in the chamber as described above, shortened by

Table 1 Average intensity ratios ($I_{1,0}/I_{1,1}$) at different physiological conditions

	TV-detector	Scintillation counter
Resting (before tetanus)	2.39 ± 0.13 (5)	2.26 ± 0.23 (6)
Rigor	0.36 ± 0.11 (5)	0.31 ± 0.04 (4)
Tetanus	0.51 ± 0.06 (6)	
Resting (after tetanus)	1.65 ± 0.17 (5)	

The intensity of each reflection was assessed from the densitometer trace or from the intensity measurements from a scintillation counter, by measuring the area under each peak. Values are means ± s.d. Number of experiments in parentheses. In each experiment, the measurement of the area was carried out for both the left and the right half of the densitometer trace (Figs 1–3 show only the right halves of the trace).

3–7% while developing the maximum tetanic tension. To compensate for this deviation from the isometric condition, the muscles for the X-ray experiments were stretched by 5% before the tetanic stimulation. The muscles produced the equatorial patterns when exposed to X rays for 10 s during tetanus, but did not sustain the maximum tension (P_0) for the length of the exposure; the tension started to decline at 5–8 s after the onset of tetanus. We used the patterns from the specimens which sustained more than 0.80 P_0 at the end of the exposure. In these “selected” specimens, the tension returned to the resting level on cessation of the stimuli. Figure 3 is a densitometer trace of the equatorial pattern thus obtained. The 1,1 reflection was intensified during tetanus; the intensity ratio (third row of Table 1) was close to that of the muscle in rigor. A Fourier analysis similar to that described by Haselgrove and Huxley⁶ showed that 86–89% of the myosin projections were in the vicinity of the thin filaments during tetanus. This is a far higher percentage than that obtained by Haselgrove and Huxley⁶ for contracting muscles (52–58%). A part of this difference might have been caused by the fact that we used a long tetanus whereas they used short tetani; some rigor-like links might have been formed between myosin and actin during a long tetanus, increasing the number of myosin projections around the thin filaments. The presence of such links was detected in separate experiments as follows. The muscle was set at a sarcomere length

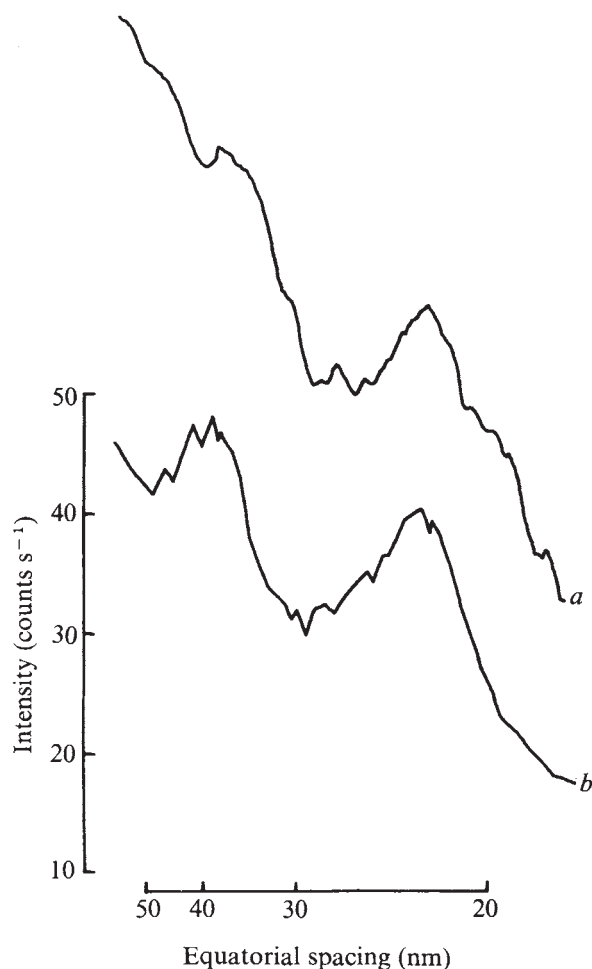


Fig. 2 *a*, A densitometer trace of the equatorial reflections recorded from a muscle in rigor at a sarcomere length of 2.2 μm . The muscle was put into rigor by soaking it for a day in Ringer's solution containing 1 mM iodoacetate. The conditions for recording were the same as in Fig. 1*a*. The 1,1 reflection was stronger than the 1,0 reflection. Note that the 1,0 peak is broad, ranging from 50 to 30 nm. *b*, Intensity distribution obtained from the same specimen using a scintillation counter.



Fig. 3 The equatorial reflections from a sartorius muscle during a 10-s tetanus. The muscle was stimulated with supra-maximal rectangular pulses (1-ms duration, 100 Hz) through a pair of platinum electrodes placed parallel to the muscle axis. The muscle length had been increased by 5% before the stimulation so that the sarcomere length became approximately 2.2 μm during tetanus. The pattern resembled that of a muscle in rigor.

of 2.2 μm , and was tetanised for 10 s. From 10 s after the end of the tetanus, the muscle was exposed to X rays for 10 s to produce a “post-tetanus” pattern. The result (fourth row of Table 1) indicated that the intensity ratio was smaller than that observed before tetanus. A Fourier analysis showed that 25–26% of the myosin projections stayed in the vicinity of the thin filaments after the tetanus.

The presence of the rigor-like links during a prolonged tetanus, even if their fraction is very great, does not affect the conclusion that most of the myosin projections are in the vicinity of the thin filaments while undergoing cyclic reactions with actin. Let us suppose that as many as 30% (>25–26%) of the projections stay in the rigor complex throughout the 10-s tetanus. Then 70% (100–30) of the projections are undergoing the cyclic reaction, and at least 56% (86–30) are in the vicinity of the thin filaments during the reaction. This still leads us to conclude that at least 80% (56/70) of the “cycling” projections are in the vicinity of the thin filaments when the muscle is producing the maximum isometric tension.

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- 1 Dupont, Y., Gabriel, A., Chabre, M., Gulik-Krzywicki, T., and Schechter, E., *Nature*, **238**, 331–333 (1972).
- 2 Chabre, M., and Cavaggioni, A., *Nature new Biol.*, **244**, 118–120 (1973).
- 3 Hashizume, H., Kohra, K., and Oguro, Y., *J. appl. Cryst.* (in the press).
- 4 Huxley, H. E., *Proc. R. Soc., B141*, 59–62 (1953).
- 5 Huxley, H. E., *J. molec. Biol.*, **37**, 507–520 (1968).
- 6 Haselgrove, J. C., and Huxley, H. E., *J. molec. Biol.*, **77**, 549–568 (1973).

Ultrastructural demonstration and analytical application of extrajunctional receptors of denervated human and rat skeletal muscle fibres

WE describe a new approach for distinguishing denervated from normal skeletal muscle fibres, based on the visualisation of differences in distribution of acetylcholine (ACh) receptors in the sarcolemma. Ionophoretic application of ACh^{1,2} and autoradiography with labelled α -bungarotoxin (α BuTX)^{3,4} have shown that after denervation (and before innervation⁵) ACh receptors appear throughout the sarcolemmal membrane, in contrast to their normal location in the neuromuscular junction. Using an immunoperoxidase staining technique^{6,7} to localise α BuTX, which binds specifically to ACh receptors^{3,4,8}, we now have a rapid and convenient method for high resolution, at light and electron microscopic levels, of the ACh receptors present throughout the sarcolemmal membrane of defectively innervated human and rat skeletal muscle fibres. We have used this immunoperoxidase method of molecular probing to analyse muscle biopsies in various human neuromuscular diseases and animal models.

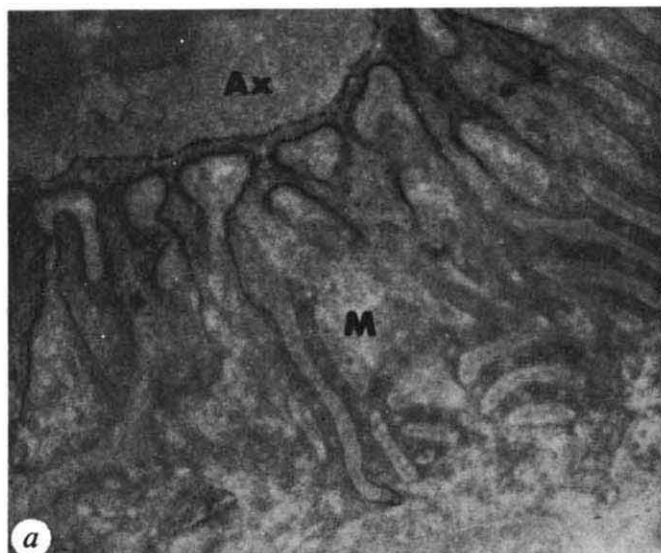
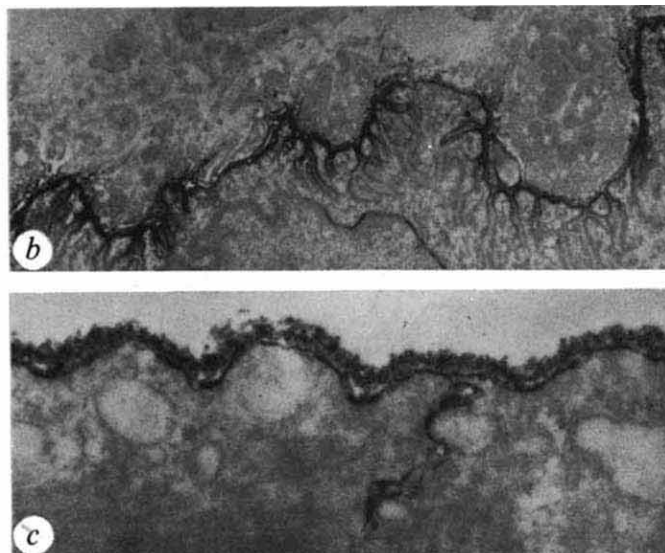


Fig. 1 ACh receptor localised by immunoperoxidase staining (black precipitate) of α BuTX binding. No counterstain was used. *a*, Normal human neuromuscular junction showing staining present at the peaks of the junctional membrane folds of the muscle fibre (M), and also on the junctional membrane of the terminal axon (Ax). (Electron micrograph $\times 23,500$.) *b*, Normal rat diaphragm neuromuscular junction with staining similar

Spurr or Epon 812. Sections ($1\ \mu\text{m}$ and $70\ \text{nm}$) were examined by light and electron (AEI EM 68) microscopy.

In normal human and rat skeletal muscle, staining consisted of an even precipitate sharply localised to the neuromuscular junction (Fig. 1*a* and *b*). Most of the α BuTX binding was localised on the peaks of the post-junctional folds, as described in other species^{6,11}. Slight additional stain was seen in the synaptic cleft as well as in a precise deposition along the presynaptic membrane. This could have been the result of either diffusion of peroxidase reaction product or actual presynaptic α BuTX binding. If the presynaptic binding of α BuTX was not a diffusion artefact, it would agree with Koelle's proposal of ACh receptors on the presynaptic membrane of the neuromuscular junction¹² as well as being an explanation of the suggested presynaptic aspect of (+)-tubocurarine action¹³. The extrajunctional sarcolemma (that is, plasmalemma) was unstained (Fig. 1*a*), except for a scant end product within $5\ \mu\text{m}$ of the junction, which may represent small numbers of ACh receptors known from ACh sensitivity studies to exist in the parajunctional sarcolemma¹⁴. In the rat diaphragm fibres, there was consistent but unexplained staining in the perinuclear membrane of nuclei in the immediate subjunctional region (Fig. 1*b*); intervening myofibrils, mitochondria and sarcoplasmic reticulum were not stained. The perinuclear staining was not seen in



to the human. Some staining is also present in the perinuclear membrane immediately below the junction (bottom centre). (Electron micrograph $\times 11,000$.) *c*, Denervated rat diaphragm muscle fibre showing intense staining of both the plasma and basement membranes. The membrane of a t-tubule running perpendicular to the plasma membrane is also stained. (Electron micrograph $\times 36,000$.)

NIH strain rats (150–200 g) were anaesthetised with an intraperitoneal injection of sodium pentobarbital. The left phrenic nerve was hooked through a thoracic stab wound and transected. At intervals of 2–45 d, after denervation, animals were killed and the entire diaphragm was removed and prefixed for 2 h in cold 2% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.4. Fibres were teased apart and incubated at 20°C in $5 \times 10^{-8}\ \text{M}$ α BuTX. After fixation in a cold periodate-lysine-paraformaldehyde solution⁹, they were incubated successively at 20°C in rabbit antibody to α BuTX and peroxidase-conjugated goat anti-rabbit immunoglobulin⁷ and post-fixed in cold 2% glutaraldehyde. Fibres were stained with 3,3'-diaminobenzidine ($0.1\ \text{mg}\ \text{ml}^{-1}$) and H_2O_2 (0.001%) (modification of procedure of Graham and Karnovsky¹⁰) and treated in 1% OsO_4 for 1 h. Specimens were embedded in low viscosity

human specimens or in rat specimens with α BuTX omitted.

In denervated rat diaphragm the muscle fibres showed the progressive morphological changes known to occur with denervation¹⁵. The extrajunctional sarcolemma was stained sharply along the length of the fibre (Fig. 1*c*) and could be distinguished clearly from that of an unstained normally innervated fibre. This spread of receptor way seen as early as 3–4 d, reaching a maximum at 14–21 d after denervation. After 3–4 weeks, the staining intensity declined. By 40 d only scant extrajunctional staining was seen, while the former junctional region remained darkly stained. The basement membrane adjacent to the stained sarcolemma was also stained; whether or not this was caused by diffusion of end product is yet to be resolved. Occasionally, stain was located on membranes of t-tubules for a short distance after their entry into the muscle fibre,

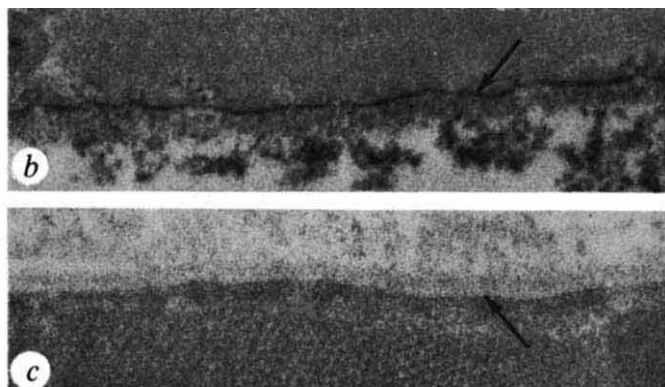
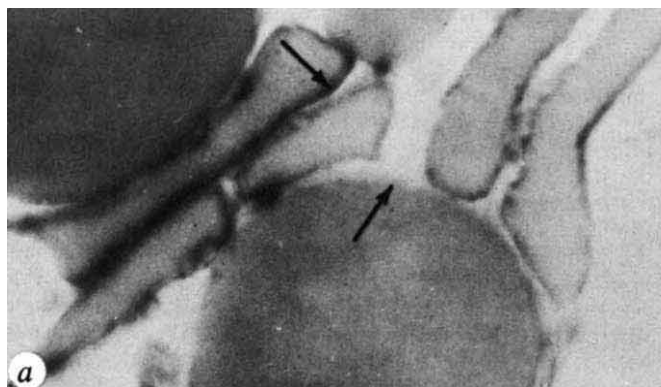


Fig. 2 Human muscle fibres in cross section from a patient with lower motor neurone disease. *a*, Group of small angular (denervated) fibres with distinct dark membrane staining (upper arrow) and two large round normal-appearing fibres with unstained membranes (lower arrow); (light micrograph $\times 675$).

Lower: periphery of one of the denervated fibres shown in (*a*). The plasma membrane ($\longrightarrow$) is darkly stained and the basement membrane only faintly stained. (Electron micrograph $\times 46,000$.) *c*, Normal-appearing fibre adjacent to that in (*b*). Plasma membrane ($\longrightarrow$) is unstained. (Electron micrograph $\times 46,000$.)

usually in regions of most intense sarcolemmal staining (Fig. 1c).

In human muscle biopsied from patients with clinical and electromyographic evidence of lower motor neurone diseases (Fig. 2*a*, *b* and *c*), staining similar to that of the denervated rat was seen throughout the sarcolemma of small fibres with angular cross-sectional contours. In serial transverse sections, these fibres were considered to be denervated according to various histochemical criteria¹⁶. There was some staining of the basement membrane. Extrajunctional staining was not seen on histochemically normal fibres in the same biopsies (Fig. 2*a* and *c*).

With α BuTX or either antibody omitted, no staining was seen in normal or denervated human or rat specimens.

The α BuTX immunochemical method can identify denervated skeletal muscle fibres with defective innervation and has provided us with a new approach to the analysis of human neuromuscular diseases. We are using this method to search for an ACh receptor blocking factor in sera of patients with myasthenia gravis. When we used human muscle biopsies containing either normal neuromuscular junction or denervated muscle fibres, sera of 75% of the patients showed the presence of such a blocking factor (our unpublished results). This method also provides a probe for altered innervation states in some patients with diagnostically uncertain clinical syndromes, for example, facioscapulohumeral and limb-girdle syndrome, as well as in certain unusual neuromuscular disorders generally thought to be myopathic but recently postulated to be caused by subtle defects in innervation¹⁶.

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- ¹ Axelsson, J., and Thesleff, S., *J. Physiol., Lond.*, **147**, 178–193 (1959).
- ² Miledi, R., *J. Physiol., Lond.*, **151**, 1–23 (1960).
- ³ Lee, C. Y., Tseng, L. F., Chiu, T. H., *Nature*, **215**, 1177–1178 (1967).
- ⁴ Hartzell, H. C., and Fambrough, D. M., *J. gen. Physiol.*, **60**, 248–262 (1972).

- ⁵ Diamond, J., and Miledi, R., *J. Physiol., Lond.*, **162**, 393–408 (1962).
- ⁶ Daniels, M. P., and Vogel, Z., *J. Cell Biol.*, **63**, 76a (1974).
- ⁷ Daniels, M. P., and Vogel, Z., *Nature* (1975).
- ⁸ Cheng, C. C., and Lee, C. Y., *Archs int. Pharmacodyn. Ther.*, **144**, 241–257 (1963).
- ⁹ McLean, J. W., and NaKane, P. K., *J. Histochem. Cytochem.*, **22**, 1077–1083 (1974).
- ¹⁰ Graham, R. C., Jr., and Karnovsky, M. J., *J. Histochem. Cytochem.*, **14**, 291–302 (1966).
- ¹¹ Fertuck, H. C., and Salpeter, M. M., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1376–1378 (1974).
- ¹² Koelle, G. B., in *Handbuch der Experimentellen Pharmakologie*, 263 (Springer, Berlin, 1963).
- ¹³ Standaert, F. G., and Riker, W. F., *Ann. N.Y. Acad. Sci.*, **144**, 517–533 (1967).
- ¹⁴ Katz, B., and Miledi, R., *J. Physiol., Lond.*, **170**, 379–387 (1964).
- ¹⁵ Pellegrino, C., and Franzini, C., *J. Cell Biol.*, **17**, 327–349 (1963).
- ¹⁶ Engel, W. K., and Warmolts, J. R., in *New Developments in Electromyography and Clinical Neurophysiology* (edit. by Desmedt, J. E.), 141 (Karger, Basel, 1973).

Subsensitivity of noradrenaline-stimulated cyclic AMP accumulation in brain slices of *d*-amphetamine-treated mice

BOTH noradrenaline (NA) and dopamine are able to stimulate the synthesis of cyclic AMP in brain tissues *in vitro*^{1,2}. Furthermore, several iontophoretic studies have indicated that the hyperpolarisation elicited by catecholamines in cerebellum³, hippocampus⁴, striatum⁵ and cortex⁶ is mediated by cyclic AMP, suggesting that this nucleotide may act as a second messenger for some actions of the catecholamines at central synapses.

It has been shown that treatments with agents like 6-hydroxydopamine^{7,8} or reserpine⁹, which result in an impaired stimulation of catecholamine receptors, enhanced the responsiveness of the catecholamine-sensitive adenylyl cyclase of brain slices or homogenates. This effect parallels the well known electrophysiological and behavioural supersensitivity observed in such conditions^{5,8}.

We report here that treatment of mice with *d*-amphetamine, a drug known to induce an overstimulation of noradrenergic receptors¹⁰, conversely results in a decreased accumulation of cyclic AMP elicited by NA in cortical slices.

Male Swiss albino mice (15–20 g; Janvier, Le Genest, France) were housed in groups of four, in a well ventilated room at an ambient temperature of 22 °C and under artificial illumination (light between 0800 and 2000). Food was available *ad libitum*. *d*-Amphetamine tartrate (100 μ g ml⁻¹) was given in the drinking water together with saccharose (2%). Control mice were also given saccharose in the drinking water. To obtain a precise initiation of drug intake, the animals were deprived of water 1 d before the beginning of the treatment. Drug consumption was evaluated by weighing the bottles. At various times after the beginning of drug intake, the mice were stunned, decapitated

Table 1 Accumulation of cyclic AMP induced by NA in cortical slices at various time intervals during treatment with *d*-amphetamine

Duration of treatment	Basal level pmol per mg protein	NA (100 μ M)	% Control stimulation
0 (41)	13.9 $\pm$ 0.7	79.6 $\pm$ 4.3	100
5 h (6)	17.1 $\pm$ 1.6	83.1 $\pm$ 5.3	76 $\pm$ 3*
12 h (12)	14.1 $\pm$ 1.1	68.8 $\pm$ 5.4	77 $\pm$ 3†
60 h (9)	14.0 $\pm$ 1.3	60.5 $\pm$ 5.5	82 $\pm$ 4*
6 d (17)	13.2 $\pm$ 0.9	55.3 $\pm$ 5.1	79 $\pm$ 3†
10 d (6)	10.9 $\pm$ 1.5	47.9 $\pm$ 6.8	71 $\pm$ 6†

At various time intervals after treatment with *d*-amphetamine the cortical slices were incubated with or without 100 μ M NA. Values are means $\pm$ s.e.m. Number of pools indicated in brackets. Control stimulation values are expressed as percentage maximal stimulation of control mice in each experiment.

* $P < 0.05$ and † $P < 0.005$ from corresponding controls.

and their brains removed and dissected on ice. Cortical slices (250 μ m thick) were prepared as described previously¹¹. Usually, the slices from two to three animals were pooled together, washed and preincubated at 37 °C for 40 min in a Dubnoff metabolic shaker under a constant stream of O₂/CO₂ (95:5). After washing, the slices were distributed in test tubes (about 5 mg of tissue per tube), gassed and further preincubated for 10 min. The incubation medium was then supplemented with appropriate test agents, the incubation was continued and cyclic AMP determined by the protein kinase binding method¹¹. Preliminary experiments had shown that NA-induced cyclic AMP accumulation was maximal between 15 and 30 min of incubation and basal level remained approximately constant until 30 min; therefore, in all further experiments, the incubations were continued for 20 min.

d-Amphetamine produced an immediate hypodipsic effect as evidenced by the water consumption during the first 12 h (1.2 $\pm$ 0.3 ml per mouse as compared with 5.6 $\pm$ 0.3 ml in control mice) corresponding to a drug intake of 6 mg kg⁻¹.

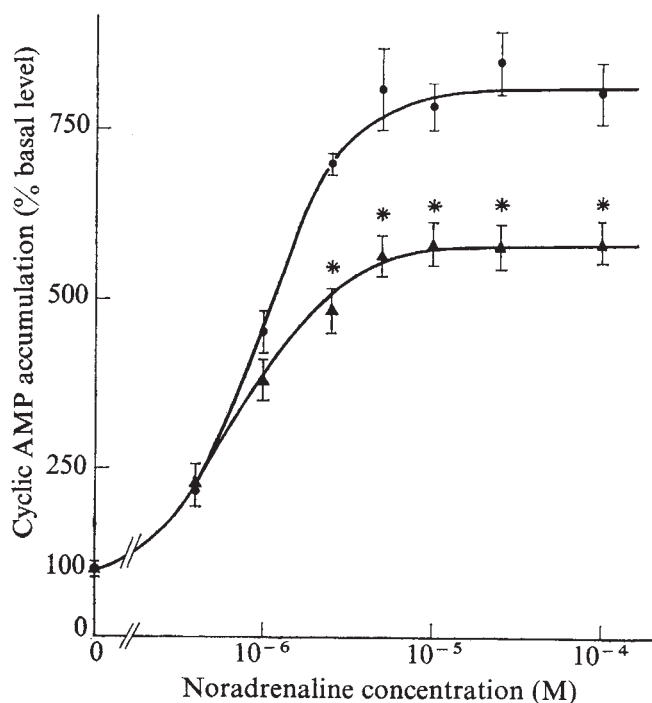


Fig. 1 Effect of noradrenaline on cyclic AMP accumulation in cortical slices of mice treated with *d*-amphetamine for 6 d. (▲) ●, Controls. Results (mean $\pm$ s.e.m.) are expressed as percentage respective basal levels (14.7 $\pm$ 0.8 pmol per mg protein for control and 16.7 $\pm$ 1.7 pmol per mg protein for *d*-amphetamine). Reciprocal plot of Dixon (S against S/V) was used for $V_{\max}$ determination and gave straight lines ($r = 0.99$ and $r = 0.99$). Eadie plot (V against V/S) was used for K_m determination ($r = 0.64$ and $r = 0.68$). * $P < 0.01$.

The water consumption progressively increased, however, during the following days so that drug intake reached 9 mg kg⁻¹ on day 6. The accumulation of cyclic AMP induced in cortical slices by a supramaximal concentration of NA (100 μ M) was determined at various time intervals after the initiation of drug treatment (Table 1). The response to NA was already significantly smaller in slices from 5 h-treated mice and there was a slight further decrease after a 10-d treatment. Concentration-response curves for the amine were determined in mice treated with *d*-amphetamine for 6 d (Fig. 1). NA-induced accumulation of cyclic AMP in cortical slices apparently followed Michaelis kinetics as the curves are well fitted with the hyperbola of Michaelis-Menten. This allows the determination, with diverse linear plots, of apparent $V_{\max}$ and K_m . Treatment with *d*-amphetamine resulted in a significant modification of the $V_{\max}$ (847 $\pm$ 26% in control against 578 $\pm$ 23% in treated mice), whereas the apparent K_m was not significantly changed (1.2 $\pm$ 0.6 μ M in control against 0.8 $\pm$ 0.4 μ M in treated mice).

To test the specificity of the effect of *d*-amphetamine treatment, the cyclic AMP accumulation induced by different agents was evaluated. Dopamine and 5-hydroxytryptamine, however, elicited only a small stimulation (Table 2) and no significant modification in responsiveness could be detected in the present conditions. In contrast, adenosine markedly activated the cyclic AMP accumulation and, after 6 d of *d*-amphetamine treatment, the response to this agent was reduced by about 25%, although this decrease was not significant.

Table 2 Accumulation of cyclic AMP elicited by various effectors in cortical slices of *d*-amphetamine-treated mice

Effector	Control (%)	<i>d</i> -Amphetamine (%)
Noradrenaline	(6) 771 $\pm$ 42	(9) 585 $\pm$ 19*
Dopamine	(6) 163 $\pm$ 15	(9) 154 $\pm$ 7
5-Hydroxytryptamine	(6) 193 $\pm$ 9	(9) 174 $\pm$ 7
Adenosine	(6) 1944 $\pm$ 185	(9) 1551 $\pm$ 111

* $P < 0.001$.

Values are mean $\pm$ s.e.m. Number of assays is indicated in brackets. Cyclic AMP accumulation induced by various effectors (100 μ M) was measured in cortical slices of mice treated with *d*-amphetamine for 6 d. Results are expressed as percentage respective basal levels: 18.4 $\pm$ 2.1 pmol per mg protein for controls and 13.7 $\pm$ 1.2 pmol per mg protein for *d*-amphetamine.

Thus, after treatment with *d*-amphetamine, the NA-induced accumulation of cyclic AMP in cortical slices is decreased, an effect which seems to be relatively specific to NA. Such an effect does not seem to be related to the presence of *d*-amphetamine in the slices as the addition of this drug in the incubation medium (at 1 μ M) does not affect either the basal level or the NA-induced accumulation of cyclic AMP. Moreover, *d*-amphetamine elicited alterations in the mechanisms of NA inactivation (through inhibition of monoamine oxidase activity or a reuptake process) cannot account for the decreased $V_{\max}$: they would presumably only decrease the apparent K_m by potentiating the effects of low concentrations of NA. A non-specific alteration of phosphodiesterase activity also seems unlikely as the stimulation induced by dopamine or 5-hydroxytryptamine is not altered at a time when sensitivity to NA is modified. In the same conditions, the response to adenosine is reduced, although not significantly. This may not be totally unexpected considering that adenosine and NA interact with a common cyclic AMP-generating compartment^{12,13}. It is not clear whether the subsensitivity to NA, induced by treatment of mice with *d*-amphetamine, is related to the catecholamine refractoriness observed in brain slices¹⁴ or cultured glioma cells¹⁵, after successive additions of the agonist, a phenomenon implicating activation of phosphodiesterase and/or rapid protein synthesis.

The NA-sensitive cyclic AMP generating system is postulated to be composed of a receptor, a transducer, a catalytic unit and, possibly, a specific phosphodiesterase¹⁶. The results of the present study do not allow us to determine which step in this complex model is modified by *d*-amphetamine treatment. As is the case after 6-hydroxydopamine⁷ or reserpine⁹ treatment, however, the modification of the sensitivity to NA is associated with a modification of the apparent $V_{\max}$ rather than with an altered K_m . This suggests that a common regulatory mechanism could be responsible for these changes, implying that the state of sensitivity of the system to NA may be modified in opposite directions according to the tonic release of the mediator.

Such a mechanism could have many implications, especially if one assumes that the sensitivity to NA of the cyclic AMP-generating system *in vitro* accurately reflects the state of responsiveness of noradrenergic postsynaptic receptors *in vivo*. First, this modified responsiveness may potentially represent a postsynaptic mechanism modulating the synaptic transmission in response to increased or decreased presynaptic activity. Such an adaptive mechanism has been already postulated to account for the modified sensitivity of the adrenergic receptor in the pineal gland, following the diurnal fluctuations of sympathetic nerve activity^{17,18}. Secondly, a decreased responsiveness to NA after *d*-amphetamine treatment may be partly responsible for the 'tolerance' to some central actions of this drug. This hypothesis, which does not exclude the participation of presynaptic mechanisms such as the formation of a false mediator or the depletion of NA stores^{19,20}, fits well with the theoretical model of tolerance involving a modified number of receptors²¹. The unchanged responsiveness to dopamine after *d*-amphetamine treatment may tentatively be related to the absence of tolerance to some central effects of this drug. No firm conclusions, however, can be drawn in this respect considering the small stimulation elicited by dopamine as well as the low drug dosage the effects of which on cortical dopaminergic neurones²² are not yet known. Finally, the decreased responsiveness to NA, if it persists after drug withdrawal, may well account for the post-amphetamine depression²³ and the associated psychological disturbances observed in humans²⁴. Work is in progress to test this hypothesis.

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Spermine inhibits induction of ornithine decarboxylase by cyclic AMP but not by dexamethasone in rat hepatoma cells

POLYAMINE biosynthesis is one of the earliest events occurring during tissue growth, and the polyamines have been implicated in the control of both RNA and protein synthesis. Increased polyamine synthesis reflects a rapid elevation of the activity of ornithine decarboxylase (ODC), the rate-limiting enzyme in the biosynthesis of the polyamines spermidine and spermine^{1,2}. Fast growing tissues exhibit the highest ODC activity, whereas in slowly growing or in non-growing tissues ODC activity is very low. ODC has been induced by various agents that affect growth processes, such as by glucocorticoids *in vivo*^{3,6}, and by cyclic AMP both *in vivo*^{6,7} and in cultured baby hamster kidney cells⁸. There have also been conflicting reports about the efficacy of polyamines in inhibiting the induction of ODC *in vivo*⁹⁻¹¹ and in activated human lymphocytes¹². As most results reported so far are from whole animal studies, we felt that a more precise understanding of ODC induction

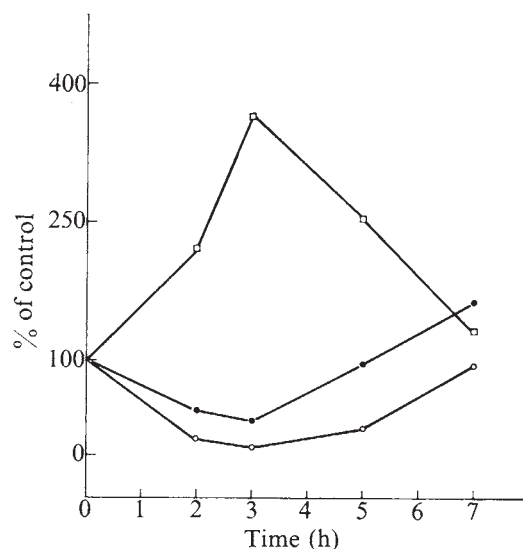


Fig. 1 Dibutyl cyclic AMP induction of ornithine decarboxylase in intact HTC cells and the effect of 10^{-5} M spermine on non-induced and cyclic AMP-induced enzyme. Hepatoma tissue culture (HTC) cells (Morris rat Hepatoma 7288C) were provided by Dr Thomas Gelehrter and were maintained in tissue culture as described previously¹⁴. Twenty four hour logarithmically growing HTC cells (at a cell density of approximately 5×10^5 cells ml^{-1}) were collected by centrifugation at 1,700g for 10 min at 24 °C in a Servall. The cells were then suspended in induction medium (growth medium from which the sera have been deleted) to the same final concentration at which they were growing. All experiments were carried out in suspension culture at 37 °C in a G77 gyratory water bath shaker (New Brunswick Scientific). N^6, O^2 -dibutyl adenosine 3',5'-monophosphate (Boehringer-Mannheim) and dexamethasone-phosphate (Merck, Sharpe and Dohme) were dissolved immediately before use in the same Tris-EDTA buffer used in the ODC assay; spermine (ICN:K & K Labs) was also prepared in this manner. At each time point, aliquots of HTC cells containing 10^7 cells ml^{-1} were centrifuged at 1,700g for 10 min at 24 °C in a Servall. For routine assays 5×10^6 cells were used per assay and the assays were always done in duplicate. The cell pellet was resuspended in assay medium, the cells broken by sonication and debris was removed by centrifugation at 20,000g for 15 min at 4 °C. The supernatant was assayed for ODC activity as described before¹⁵. The assay buffer contained 50 mM Tris (pH 7.8 at 37 °C), 1 mM EDTA, 0.5 mM pyridoxal phosphate, 5 mM dithiothreitol and non-saturating levels of 6×10^{-5} M L-1,14C-ornithine. □, Cyclic AMP (10^{-3} M); ○, spermine (10^{-5} M) or spermidine (6×10^{-5} M); ●, cyclic AMP (10^{-3} M) + spermine (10^{-5} M) or spermidine (6×10^{-5} M).

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- Forn, J., Krueger, B. K., and Greengard, P., *Science*, **186**, 1118-1120 (1974).
- Von Hungen, K., and Roberts, S., *Eur. J. Biochem.*, **36**, 391-401 (1973).
- Hoffer, B. J., Siggins, G. R., Oliver, A. P., and Bloom, F. E., in *Advances in Cyclic Nucleotide Research*, 1 (edit. by Greengard, P., and Robison, G. A.), 411-423 (Raven, New York, 1972).
- Segal, M., and Bloom, F. E., *Brain Res.*, **72**, 79-97 (1974).
- Siggins, G. R., Hoffer, B. J., and Ungerstedt, U., *Life Sci.*, **15**, 779-792 (1974).
- Stone, T. W., Taylor, D. A., and Bloom, F. G., *Science*, **187**, 845-847 (1975).
- Kalisker, A., Rutledge, C. O., and Perkins, J. P., *Molec. Pharmacol.*, **9**, 619-629 (1973).
- Mishra, R. K., Gardner, E. L., Katzman, R., and Makman, M. H., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3883-3887 (1974).
- Dismukes, K., and Daly, J. W., *Molec. Pharmacol.*, **10**, 933-940 (1974).
- Glowinski, J., and Baldessarini, R. J., *Pharmac. Rev.*, **18**, 1201-1238 (1966).
- Baudry, M., Martres, M. P., and Schwartz, J. C., *Nature*, **253**, 362-363 (1975).
- Schultz, J., and Daly, J. W., *J. Neurochem.*, **21**, 1319-1326 (1973).
- Skolnick, P., and Daly, J. W., *J. Neurochem.*, **24**, 451-456 (1975).
- Schultz, J., and Daly, J. W., *J. Biol. Chem.*, **248**, 860-866 (1973).
- De Vellis, J., and Brooker, G., *Science*, **186**, 1221-1223 (1974).
- Perkins, J. P., in *Advances in Cyclic Nucleotide Research*, 3, (edit. by Greengard, P., and Robison, G. A.), 1-64 (Raven, New York, 1973).
- DeGuchi, T., and Axelrod, J., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 2411-2414 (1973).
- Romero, J. A., and Axelrod, J., *Science*, **184**, 1091-1092 (1974).
- Brodie, B. B., Cho, A. K., and Gessa, G. L., in *Proc. Int. Symp. Amphetamines related compounds* (edit. by Costa, E., and Garattini, S.), 219-230 (Raven, New York, 1970).
- Lewander, T., *Psychopharmacologia, Berl.*, **21**, 17-31 (1971).
- Kalant, H., Leblanc, A. E., and Gibbins, R. J., *Pharmac. Rev.*, **23**, 135-191 (1971).
- Thierry, A. M., Blanc, G., Sobel, A., Stinus, L., and Glowinski, J., *Science*, **182**, 499-501 (1973).
- Tonge, S. R., *Psychopharmacologia, Berl.*, **38**, 181-186 (1974).
- Jönsson, L. E., and Gunne, L. M., in *Proc. Int. Symp. Amphetamines related compounds*, (edit. by Costa, E., and Garattini, S.), 929-936 (Raven, New York 1970).

may result from investigation of an isolated system. The system chosen for detailed study was rat hepatoma cells (HTC) because most *in vivo* studies have focused on ODC from rat liver. We now report the induction of ODC in HTC cells by both cyclic AMP and dexamethasone.

Induction by cyclic AMP reached a maximum fourfold increase after about 3 h, and activity declined to basal levels by 7 h (Fig. 1). Induction by dexamethasone, on the other hand, reached a sixfold increase in activity by 12 h and remained at this level for more than 20 h in the presence of the inducing agent (Fig. 2). The difference between the two types of induction was investigated using spermine and inhibitors of RNA and protein synthesis. When spermine was added to the induction medium simultaneously with the inducing agent, 10^{-5} M spermine was sufficient not only to abolish the cyclic AMP induction, but also to depress ODC activity well below control level (Fig. 2). Spermidine at 6×10^{-5} M was equally effective as spermine. When ODC was induced by dexamethasone, neither spermidine nor spermine had any effect, even at concentrations of up to 10^{-2} M (Fig. 2).

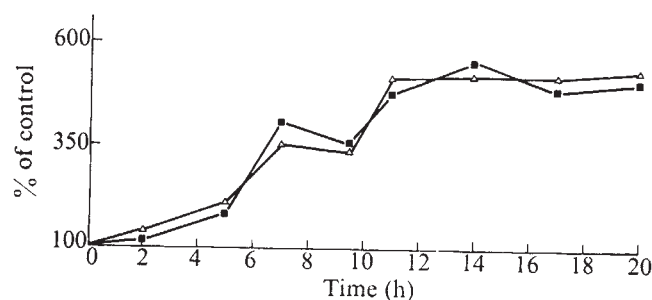


Fig. 2 Dexamethasone induction of ornithine decarboxylase in intact HTC cells and the effect of 10^{-5} M spermine on the induced enzyme. The conditions of the experiment are identical to those described for Fig. 1. Δ , Dexamethasone phosphate (2×10^{-6} M); $\blacksquare$, dexamethasone phosphate (2×10^{-6} M) + spermine (10^{-5} M) or spermidine (6×10^{-5} M).

To time the inhibition by spermine of the induction of ODC, cyclic AMP and spermine were added simultaneously; spermine was then removed at different times by centrifuging the cells and resuspending them in induction medium containing that concentration of cyclic AMP present initially. In approximately 30 min (the time required for handling the samples), 10^{-5} M spermine completely inhibited induction by cyclic AMP. This rapid depression of ODC activity suggests a functional role for spermine in its control of ODC.

To investigate the relative effectiveness of spermine and inhibitors of RNA and protein synthesis with respect to induction of ODC, we added 10^{-5} M spermine or cycloheximide ($25 \mu\text{g ml}^{-1}$) or actinomycin D ($5 \mu\text{g ml}^{-1}$) at the same time as cyclic AMP or dexamethasone; ODC activity was then measured after 3 h for cyclic AMP and 15 h for dexamethasone. Table 1 shows that neither cycloheximide nor actinomycin D affected the basal enzyme level, whereas spermine inhibited it by 50%; furthermore, spermine was unique in affecting only cyclic AMP induction. Cycloheximide abolished induction by both agents; although actinomycin D completely inhibited induction by dexamethasone it only partially (65%) inhibited induction by cyclic AMP.

The low effective concentration of spermine, its speed of action, as well as its selectivity in inhibiting only the induction by cyclic AMP precludes feedback inhibition as the possible mechanism of its action. The cyclic GMP-cyclic AMP ratio and the spermine-spermidine ratio are considered to be important factors in regulation of ODC synthesis¹³. Results with cycloheximide verify the role of

Table 1 Ornithine decarboxylase activity (p mol per mg protein per 30 min)

	Control	Cyclic AMP (10^{-3} M)	Dexamethasone (2×10^{-6} M)
Control	130 ± 5	536 ± 12	806 ± 15
Spermine (10^{-5} M)	65 ± 3	78 ± 3	802 ± 21
Cycloheximide ($25 \mu\text{g} \times \text{ml}^{-1}$)	130 ± 7	130 ± 2	128 ± 3
Actinomycin D ($5 \mu\text{g} \times \text{ml}^{-1}$)	130 ± 4	182 ± 3	130 ± 4

The effects of spermidine, cycloheximide, and actinomycin D on cyclic AMP-induced ornithine decarboxylase in whole HTC cells. The conditions of the experiment are those described for Fig. 1. Spermine, cycloheximide (Sigma) and actinomycin D (Merck, Sharpe and Dohme) were added at the time of addition of the inducing agent. The effects of these compounds were measured at 3 h for the cyclic AMP-induced ODC and at 15 h for the dexamethasone induced ODC enzyme.

protein synthesis in induction of ODC. Continuous protein synthesis may not be necessary for basal ODC activity as this is not inhibited by cycloheximide; that this basal activity is affected by spermine suggests a unique role for this agent. The only partial inhibition by actinomycin D, which is observed also in cultured baby hamster kidney cells⁸, indicates a post-transcriptional site of action for cyclic AMP in contrast to that of dexamethasone. The ability of ODC to fluctuate rapidly in response to the introduction of these agents suggests that elevation of ODC activity may be an important step in the control of cellular activity.

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- Pegg, A. E., and Williams-Ashman, H. G., *Biochem. J.*, **108**, 533-539 (1968).
- Williams-Ashman, H. G., Pegg, A. E., and Lockwood, D. H., *Adv. Enzyme Regul.*, **7**, 291-323 (1969).
- Richman, R. A., Underwood, L. E., Van Wyk, J. J., and Voina, S. J., *Proc. Soc. exp. Biol. Med.*, **138**, 880-884 (1971).
- Rizzo, A. J., Heilpern, P., and Webb, T. E., *Cancer Res.*, **31**, 876-881 (1971).
- Cavia, E., and Webb, T. E., *Biochim. Biophys. Acta*, **262**, 546-554 (1972).
- Beck, W. Y., Bellantone, R. A., and Canellakis, E. S., *Biochem. Biophys. Res. Commun.*, **48**, 1649-1655 (1972).
- Hölttä, E., and Raina, A., *Acta endocr., Copenh.*, **73**, 794-800 (1973).
- Hogan, B., Shields, R., and Curtis, D., *Cell*, **2**, 229-233 (1974).
- Schrock, T. R., Oakman, N. J., and Bucher, N. L. R., *Biochim. Biophys. Acta*, **204**, 564-577 (1970).
- Jänne, J., and Williams-Ashman, H. G., *J. Biol. Chem.*, **246**, 1725-1732 (1971).
- Jänne, J., and Hölttä, E., *Biochem. Biophys. Res. Commun.*, **61**, 449-456 (1974).
- Kay, J. E., and Lindsay, V. J., *Biochem. J.*, **132**, 791-796 (1973).
- Theocharides, T. C., *Rec. Progr. Hormone Res.* (in the press).
- Gelehrter, T., Emanuel, J. R., and Spencer, C. J., *J. Biol. Chem.*, **247**, 6197-6203 (1972).
- Russell, D. H., and Snyder, S. H., *Proc. natn. Acad. Sci. U.S.A.*, **60**, 1420-1427 (1968).

Increased neural lobe content of neurophysins in rats treated with exogenous vasopressin

In a number of endocrine systems it has been established that negative feedback mechanisms are involved in the regulation of hormone synthesis and release. No such role has however, been established for vasopressin in the control of the hypothalamo-neurohypophyseal neurosecretory system. The present experiments were carried out to determine the effects of administration of exogenous vasopressin on the stores of the neurohypophyseal peptides oxytocin and vasopressin and the proteins (neurophysins) associated with them in the neurosecretory granules.

Groups of male Wistar rats (Porton strain) of approximately 200 g body weight were given daily intramuscular injections of either 0.5 IU vasopressin tannate in oil or the same volume (0.1 ml) of the vehicle alone. This dose of

Table 1 Mean ($\pm$ s.e.) content of neurohypophysial vasopressin (pressor activity), oxytocin (milk ejection activity) and neurophysins A, B and C in rats injected with vasopressin tannate in oil (0.5 IU daily intramuscularly for 8 d) or arachis oil

Injection	Vasopressin ($\times 10^{-3}$ IU)	Oxytocin ($\times 10^{-3}$ IU)	Neurophysin (arbitrary units)		
			A	B	C
Oil	234.3 $\pm$ 8.5	225.7 $\pm$ 19.1	16.8 $\pm$ 0.7	6.5 $\pm$ 0.3	3.7 $\pm$ 0.3
	NS	NS	$P < 0.02$	$P < 0.01$	$P < 0.01$
Vasopressin	227.7 $\pm$ 9.9	221.6 $\pm$ 24.7	19.5 $\pm$ 0.7	7.9 $\pm$ 0.4	5.3 $\pm$ 0.3

All values are presented in terms of metabolic size, that is, per (100 g initial body weight)^{0.75} to take into account minor differences in body weight between animals⁵. The number of figures constituting each mean varied between 11 and 19. Results were compared using Student's *t* test.

NS, not significant.

vasopressin is sufficient to raise urinary osmolality and reduce water intake significantly without causing alteration of plasma osmotic pressure values¹. After 8 d of treatment rats in the two experimental groups were killed and the neural lobes removed. Half of the glands from each group were homogenised in 0.05 M acetic acid and the extracts subsequently used for hormone bioassay. Vasopressin and oxytocin were assayed using respectively the rat vasopressor and rat milk ejection techniques. The other half of the glands were homogenised in a small volume (50 μ l) of 0.1 M HCl and subjected to polyacrylamide gel electrophoresis as described previously². After electrophoresis and a dye staining-destaining step the quantities of the three rat neurophysins (one minor and two major components) present in each gel were estimated using a densitometric method (C.W.J., unpublished) similar to that described previously³.

The results (Table 1) indicate that in these conditions of vasopressin administration the neural lobe content of vasopressin and oxytocin was unaltered when compared with control animals. In contrast, however, there was a significant increase in the neural lobe content of all three rat neurophysins.

It is difficult to explain the accumulation of neurophysins, without any associated change in hormone content, in the vasopressin-treated animals. It may be that there has been an inhibition of the release of oxytocin, vasopressin and neurophysins from the neurohypophysis accompanied by a more rapid intracellular degradation of hormones than of neurophysins. A mechanism involving intracellular degradation has been postulated by Smith and Farquar⁴, which removes excess hormone from adenohypophysial tissues. Although at present an element of speculation must remain in the interpretation of these findings, two points emerge clearly. First, either directly or indirectly, administration of high levels of exogenous vasopressin can influence the hypothalamo-neurohypophysial system. Second, the bioassayable hormone in the neurohypophysis may not always bear a constant relationship to the content of neurophysins.

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λ Lysogens of *E. coli* reproduce more rapidly than non-lysogens

THE evolutionary basis for the integration of phage DNA into bacterial chromosomes (lysogeny^{1,2}) is not understood. A generally accepted explanation is that when the bacterial population is large and growing well, the phages prefer the lytic pathway since they can multiply without elimination of all host bacteria. If conditions deteriorate so that the supply of bacteria becomes limited or if growth is slowed, the phages tend to lysogenise. There have been few studies of the evolutionary mechanisms which may have selected for lysogeny. We now report that in certain growth conditions λ lysogens of *Escherichia coli* reproduce more rapidly than isogenic, non-lysogenic strains.

E. coli, AB 257 *met*, lysogenised with a temperature inducible phage λ (C₁₈₅₇) grows normally at 30 °C but is lysed if the temperature is raised to 42 °C. Two other mutations in the phage prevent its induction or replication at 30 °C. The *ind* mutation³ reduces the inducibility of the prophage to a very low level and the *susJ* mutation⁴ makes phage production impossible in this non-permissive *E. coli* host. At 42 °C the prophage is induced by the thermolability of the C₁ repressor protein and the host bacteria are lysed.

A mixed population of the lysogenic and non-lysogenic bacteria was grown in broth or M-9 medium⁵. Fig. 1 shows that there was little change in the frequency of the bacterial types in the population in these growth conditions. The ratio of the two bacterial types fluctuated from day to day but the frequency remained relatively constant in repeated experiments. The growth rates of the separate strains in M-9 medium indicated that both strains had a generation time of 80 min in either excess or limited glucose. Figure 1 shows that in broth there was no significant change in frequency after 80 generations.

If equal mixtures of the lysogenic and non-lysogenic bacteria were grown in a chemostat in which growth was limited by the glucose concentration of 0.01%, however, lysogenic bacteria rapidly replaced non-lysogenic bacteria (Fig. 1). The rate of increase of lysogenic bacteria varied in different experiments but the lysogen usually attained a frequency greater than 99% within 30 generations. At this concentration of glucose the population density was $2\text{--}3 \times 10^8$ bacteria per ml and the flow of medium was adjusted to give 10–15 generations per day. In the chemostat

Table 1 Change in the frequency of the lysogenic *E. coli* in a mixed population after shift to a different growth medium

Shift	Generations	Lysogen frequency	
		Before	After
Broth to broth	40	0.46	0
Broth to glucose	20	0.46	
Glucose to broth	20	0.41	
Exponential to stationary	40	0.46	

¹ Bakker, H. R., and Dyball, R. E. J., *Neuroendocrinology* (in the press).

² Burford, G. D., Jones, C. W., and Pickering, B. T., *Biochem. J.*, **124**, 809 (1971).

³ Sunde, D., and Sokol, H. W., *Ann. N.Y. Acad. Sci.* (in the press).

⁴ Smith, R. E., and Farquar, M. G., *J. Cell. Biol.*, **31**, 319 (1966).

⁵ Brody, S., *Bioenergetics and Growth* (Reinhold, New York, 1945).

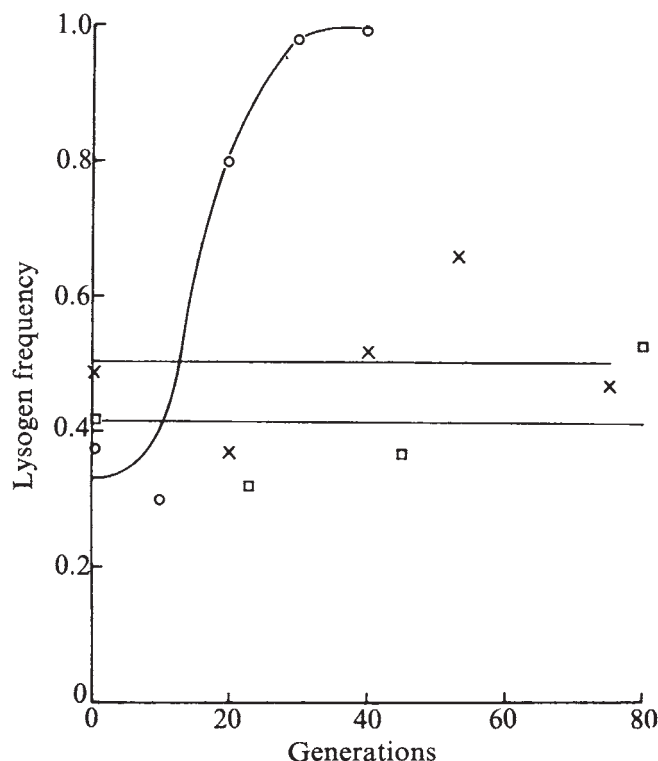


Fig. 1 Growth of a mixed population of lysogenic and non-lysogenic *E. coli* in different conditions. The lysogenic and non-lysogenic bacteria were grown separately overnight in M9 (0.2% glucose, $40 \mu\text{g ml}^{-1}$ methionine). A portion of each culture was mixed to give equal numbers of both types and the exact number of each type was determined by spreading a dilution of the mixed population on broth-agar plates and incubating duplicate plates at 30°C and 42°C . The number of colonies at 42°C gives the number of non-lysogenic bacteria and the number of colonies at 30°C gives the total number of bacteria. For the chemostat experiments the frequency of each type was measured in the initial inoculum, after overnight growth in the chemostat and at daily intervals thereafter. For the serial dilution experiments the mixed population was diluted 10^6 daily into fresh medium and grown at 30°C . The cultures were sampled daily. ○, Frequency of the lysogen in a chemostat; ×, frequency of the lysogen in M9 plus 0.4% glucose and $40 \mu\text{g ml}^{-1}$ methionine; □, frequency of the lysogen in broth.

experiment shown in Fig. 1, bacteria were grown separately overnight in 0.2% glucose and equal mixtures were diluted into a chemostat containing 0.01% glucose and grown overnight without additional medium. In other experiments each strain was grown separately overnight in 0.01% glucose and then a mixture was diluted into the chemostat. If the bacteria were adapted to 0.01% glucose, the population was converted to the lysogen more rapidly than is shown in Fig. 1.

Since the bacteria were being shifted from a medium of high glucose concentration (0.2%) to low concentration (0.01%), control experiments were done to determine the effect of abrupt changes in growth medium on the frequency of the strains in the population. Table 1 shows the results of nutritional shift-up and shift-down experiments. There is no significant change in the frequency of the two strains after shift from broth to M-9 or vice versa. In addition, the frequency of the two types was measured in exponential growth (10^7 bacteria ml^{-1}), stationary phase (10^8 bacteria ml^{-1}) and after continued incubation in stationary phase for more than 24 h. Table 1 shows that the initial and final frequency of the lysogen was unchanged throughout these changes in growth conditions.

To determine whether the lysogen strain was more fit than the non-lysogen strain in other conditions, mixed populations were grown in chemostats in which growth was

limited by glycerol, lactose or acetate (all 0.01%) or by methionine ($0.05 \mu\text{g ml}^{-1}$). Figure 2 shows the change in limited glycerol or methionine. In limited glycerol the frequency of the lysogen in the population increased rapidly, and the same increase was observed in limited lactose or acetate. Methionine limitation, however, did not significantly increase the frequency of the lysogen. In several chemostat experiments in which growth was limited by methionine, the lysogen showed no persistent increase in frequency although the data in Fig. 2 show an initial increase and subsequent decline in frequency. The experiments with methionine limitation were complicated by selection for methionine, revertants in the chemostat and it was impossible to grow the population for long periods without reversion to prototrophy. The data suggest however, that methionine limitation does not confer the same increased fitness that is evident when the carbon source is limited.

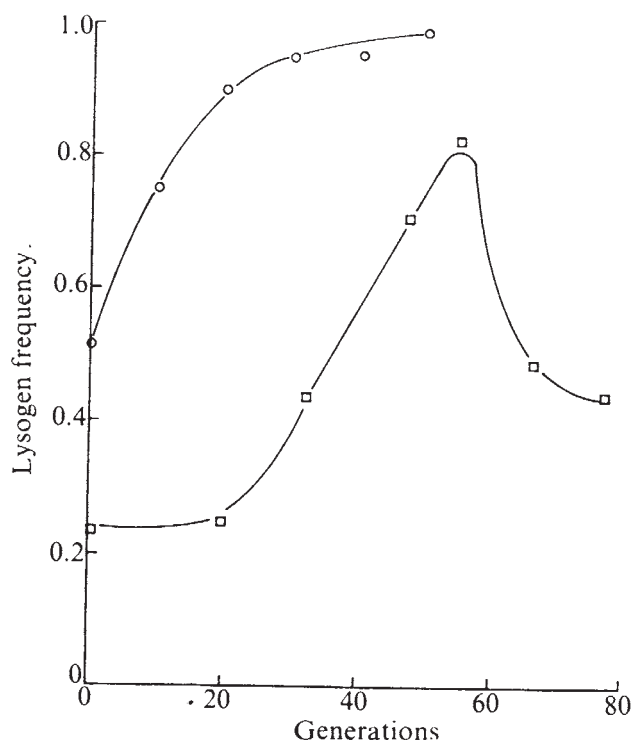


Fig. 2 Growth of a mixed population of lysogenic and non-lysogenic bacteria in limited glycerol or limited methionine in a chemostat. Growth and frequency was as described in Fig. 1. ○, Limited glycerol (0.01%) chemostat; □, limited methionine ($0.05 \mu\text{g ml}^{-1}$) chemostat.

In view of the novelty of these results, a number of control experiments were done. To eliminate the strain specificity of the phenomena, a strain of *E. coli* B which carries the λ attachment site on its DNA was also lysogenised. This lysogenic *E. coli* B strain showed the same increased reproduction rate over the non-lysogenic strain when grown in a mixed population in limited glucose.

One unexplained aspect of the increased reproduction rate of the lysogen is the final frequency reached after extended growth in the chemostat. If a dilution of the lysogenic strain alone was spread on agar plates and incubated at 30°C and 42°C , fewer than 10^{-6} bacteria formed colonies at 42°C . In all chemostat experiments performed the final frequency of non-lysogenic bacteria was never less than 10^{-4} and usually stabilised at 10^{-2} . This suggests that although the lysogen is reproduced more rapidly than the non-lysogen in the chemostat, the non-lysogen is not eliminated from the population but is maintained at a low frequency.

Some bacteria may attach to the walls of the chemostat

thus maintaining the non-lysogenic bacteria in the culture. To test this, a sample from a chemostat in which the frequency of the two bacterial types had stabilised was diluted into an identical sterile chemostat and growth continued. No further change in frequency was observed, suggesting that attachment does not maintain the non-lysogen in the culture.

So far the data indicate that an *E. coli* bacterium carrying the prophage λ is more fit than a non-lysogen when growth is limited by the carbon source. In several other conditions this increased rate of reproduction is not conferred. We are testing the generality of this increased reproduction rate with regard to other prophages and chromosome attachment sites.

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¹ Lwoff, A., *Bact. Rev.*, **17**, 269–337 (1953).

² Echols, H., *A. Rev. Genet.*, **6**, 157–190 (1972).

³ Sussman, R., and Jacob, F., *C.r. hebd. Séanc. Acad. Sci., Paris.*, **254**, 1517–1519 (1962).

⁴ Sly, W., Eisen, H., and Siminovich, L., *Virology*, **34**, 112–127 (1968).

⁵ Van Brunt, J., and Edlin, G., *J. molec. Evol.* (in the press).

Reassembly of a spherical virus in mild conditions

ALTHOUGH successful reassembly of a number of spherical viruses has been reported^{1–5}, this has hitherto only been accomplished in conditions which are clearly non-physiological. We now report the reassembly of cowpea chlorotic mottle virus (CCMV) in mild conditions, $\sim$ pH 6, ionic strength 0.1 or 0.2 and 20 to 25 °C; conditions which are plausible for an *in vivo* assembly reaction.

The virus was grown as described previously⁶ and the protein prepared by dissociation at high pH and ionic strength, followed by removal of the RNA either by calcium precipitation and ion exchange chromatography⁶ or by sedimentation of the RNA⁴. RNA for reassembly was prepared by dissociation of the virus with 2% SDS at 50 °C and then either phenol extraction or sucrose gradient purification of the RNA. Reassembly was obtained either when the protein, weakly buffered to pH 8.0 at ionic strength 0.2, was mixed with a strongly buffered RNA solution (at pH 6.0, ionic strength 0.2) to give a final pH near to 6 at 25 °C, or alternatively when the protein and RNA were mixed at pH 7.5 and then dialysed rapidly to pH 6.0, ionic strength 0.1 at 20 °C. Neither of these procedures is necessarily the best possible choice, as the optimum conditions for the reassembly have not yet been sought, but even so with an RNA–protein ratio of 1:4 or 1:5 (compared with the proportion of 1:3.3 which occurs in the virus), at least 80% of the RNA becomes encapsidated within 2 h.

The resulting reassembled material has a sedimentation coefficient of 75–78S, compared with 77–79S for the normal virus: scans from sedimentation velocity experiments are shown in Fig. 1. Reassembled particles also have a similar molecular weight to native ones of about 4.5×10^6 , as determined by sedimentation equilibrium, and a similar density in caesium chloride gradients. Electron micrographs of reassembled particles are again very similar to those of the original virus (Fig. 2) and besides the characteristic two-fold views marked in Fig. 2 (comparable with those shown for the capsid⁷) we have also seen three-fold, five-fold and local two-fold views which are similar to those found for the closely related broad bean mottle virus⁸. As a further

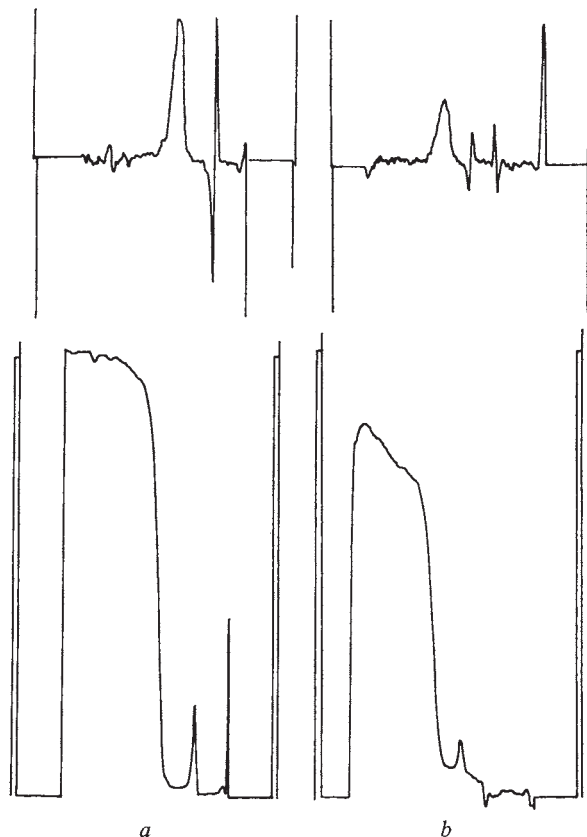


Fig. 1 Sedimentation of native (a) and reassembled (b) CCMV observed in the analytical ultracentrifuge. Samples in sodium phosphate, pH 6.0, *I* 0.2 at 20 °C centrifuged at 20,000 r.p.m. Scans at 260 nm were taken approximately 15 min after reaching full speed. Lower curves show the absorbance along each cell, with the derivative curve above.

check of the quality of the reassembled product, its infectivity was compared with that of native CCMV in a dilution assay on cowpea plants. Both produced systemic infections when inoculated at concentrations of 0.1 mg ml⁻¹ but not at 0.02 mg ml⁻¹, while the RNA used for the reassembly did not produce infection at 0.02 mg ml⁻¹ (corresponding to 0.09 mg ml⁻¹ virus). Although this is only a preliminary comparison of the infectivities, this distinction between the native and reassembled CCMV on the one hand and their isolated RNA on the other is again compatible with the resemblance of our reassembled material to native undissociated CCMV.

In the final conditions used for this reassembly, a solution of the protein alone would, at equilibrium, contain less than 10% capsid (with sedimentation coefficient around 50S) and over 90% small aggregates (around 3S)⁶, but there is a marked hysteresis on going from the disaggregated state (for example, pH 7.5, ionic strength 0.2) to the capsid⁶. This hysteresis was observed over a period of hours at both 5 and 23 °C, but equilibrium appears to be established within 48 h. Also in these conditions, an hexagonal array of CCMV protein subunits is frequently observed on the specimen grids used for electron microscopy, and we had speculated⁶ that this might be due to a nucleation barrier for the protein aggregation which is overcome on interaction with the carbon surface of the grid and that it may also be overcome by interaction with the viral RNA. But direct mixing of the equilibrium protein and RNA did not lead to the reassembly of stable virus-like particles, but only to the formation of more capsid. To obtain such reassembly, it was necessary to supply the protein in a non-equilibrium form, by taking advantage of the hysteresis as already described. The difference in the properties of equilibrium and non

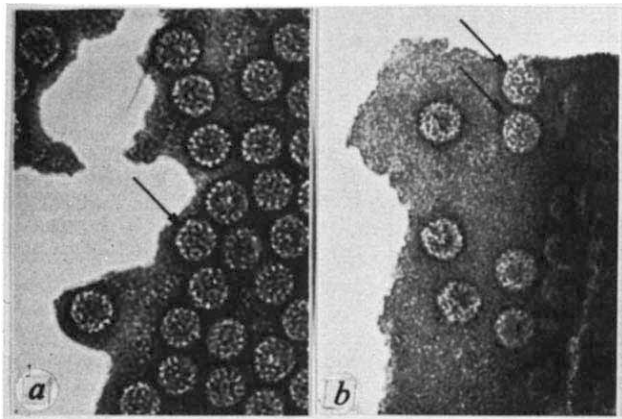


Fig. 2 Electron micrographs of (a) native and (b) reassembled CCMV. Marked particles show the twofold view characteristic of particles with this surface morphology. Samples were negatively stained with 1% (w/v) uranyl acetate and photographed over holes in the carbon substrate. ($\times 225,000$).

equilibrium protein at pH 6 is presumably due to the greater degree of aggregation of the former, while the less aggregated non-equilibrium material may be in a state closer to that of freshly synthesised coat protein in a natural infection.

The reassembly we describe is highly reproducible and occurs to a substantial extent over the range from pH 5.5 to 6.5, ionic strength 0.1 to 0.5 and temperature between 5 and 25 °C. In addition, we have not, so far, found any marked effect of magnesium or polyamine ions, in contrast to their requirement for satisfactory reassembly of CCMV from high ionic strength⁸. We have therefore found reassembly within the region of physiologically plausible conditions, although these can clearly be refined by further work. Thus, in spite of its close similarity to the native virus, our reassembled product is less stable in caesium chloride and we think that this may be due to the lack of some annealing process during or after the main assembly. The way is now open for further investigation both of this process and of the selectivity of the virus protein for its multiple RNA species.

Previously, the reassembly of spherical viruses has required the presence, and subsequent removal, of denaturing agents (such as 5.7 M urea for R17 (ref. 1) or 8 M guanidine hydrochloride for Q β (ref. 2)) or of high concentrations of salt at low temperature (for cucumber mosaic virus³ and the CCMV group^{4,5}). Clearly such reassembly departs, at least in this respect, from the normal assembly of the virus and may not even follow the same pathway. We believe that the more significant processes will be found to occur near neutrality, at ionic strengths of 0.1 to 0.2 and temperatures from 20 to 37 °C. In particular, the phenomenon of selectivity of the protein for its homologous RNA may well be best observed in such conditions, as in the case of the rod-shaped tobacco mosaic virus^{10,11}. Another similarity with the reassembly of tobacco mosaic virus lies in the requirement for the protein to be in the correct aggregation state for the optimum reaction, that is, the disk for tobacco mosaic virus¹¹ and the "hysteresis protein" for CCMV.

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¹ Roberts, J. W., and Steitz, J. E. A., *Proc. natn. Acad. Sci. U.S.A.*, **58**, 1416-1421 (1967).

- ² Hung, P. P., and Overby, L. R., *Biochemistry*, **8**, 820-828 (1969).
- ³ Kaper, J. M., and Geelen, J. L. M. C., *J. molec. Biol.*, **56**, 277-294 (1971).
- ⁴ Bancroft, J. B., and Hiebert, E., *Virology*, **32**, 354-356 (1967).
- ⁵ Hiebert, E., and Bancroft, J. B., *Virology*, **39**, 296-311 (1969).
- ⁶ Adolph, K. W., and Butler, P. J. G., *J. molec. Biol.*, **88**, 327-341 (1974).
- ⁷ Finch, J. T., and Bancroft, J. B., *Nature*, **220**, 815-816 (1968).
- ⁸ Finch, J. T., and Klug, A., *J. molec. Biol.*, **24**, 289-302 (1967).
- ⁹ Jacrot, B., *J. molec. Biol.* (in the press).
- ¹⁰ Fraenkel-Conrat, H., and Singer, B., *Virology*, **23**, 354-362 (1964).
- ¹¹ Butler, P. J. G., and Klug, A., *Nature new Biol.*, **229**, 47-50 (1971).

A complex genetic locus that controls the first three steps of pyrimidine biosynthesis in *Drosophila*

IN eukaryotes, the initial steps in the *de novo* synthesis of pyrimidines are performed by multienzyme complexes consisting of the first two enzymes in fungi and the first three enzymes in mammals^{1,2}. In *Neurospora* there is evidence that this complex facilitates channelling of the carbamyl phosphate produced by pyrimidine-specific carbamyl phosphate synthetase (CPSase) to the second enzyme, aspartate transcarbamylase (ATCase)³. The aggregation of these enzymes in fungi correlates with close linkage of the structural genes for both CPSase and ATCase which are apparently cotranscribed and translated⁴⁻⁷. In fungi the third pyrimidine pathway enzyme, dihydroorotase (DHOase) is encoded by an unlinked gene^{5,8}. Considering the close relationship between enzyme aggregation and gene clustering in this system in fungi, the presence of the first three steps of pyrimidine biosynthesis in a single mammalian enzyme aggregate led us to question whether all three structural genes may be clustered in animals. We now report that this is the case in *Drosophila* and that the fourth enzyme in the pathway is not included within this gene cluster.

The sex-linked, recessive mutation *rudimentary* (*r*) wing affects wing morphology and female fertility in *D. melanogaster*^{9,10}. Genetic complementation occurs among certain *r* alleles¹¹ and complementation maps of the locus are colinear with fine structure maps^{12,13}. Carlson has defined seven complementation units, I-VII (ref. 13) (Fig. 1). Subsequent to these detailed genetic studies, *r* flies have been shown to be auxotrophic for pyrimidines or pyrimidine precursors^{14,15}. Based on nutritional studies and direct assay of ATCase, Nørby proposed that the *r* locus controls at least the first two steps in *de novo* pyrimidine biosynthesis, CPSase and ATCase^{16,17}. Specifically, he showed that complementation unit I alleles lack ATCase and proposed that unit VI alleles lack CPSase. This hypothesis has been verified by Jarry and Falk and extended to include unit III within the ATCase zone of the *r* locus¹⁸. These workers also found normal levels of both CPSase and ATCase in flies possessing a complementation unit VII allele. From our own work we find that flies of this complementation class are deficient in activity of DHOase. This observation coincides with one of the suggestions for the function of this complementation unit independently put forward by Jarry and Falk¹⁸.

Table 1 Complementation among *r* alleles

		I-VII (<i>r</i> ^{60 d1})	VII (<i>r</i> ³⁷¹⁰)	VI (<i>r</i> ^{39 k})	I-IV (<i>r</i> ^{59 k23})
I-IV	(<i>r</i> ^{59 k23})	—	+	+	—
VI	(<i>r</i> ^{39 k})	—	+	—	—
VII	(<i>r</i> ³⁷¹⁰)	—	—	—	—
I-VII	(<i>r</i> ^{60 d1})	—	—	—	—

Alleles were combined in *trans* heterozygotes which were scored for wing phenotype. Complementation group designations are in Roman numerals while the actual allele used is noted in parentheses. These mutants are described in detail elsewhere¹³. Complementing combinations (+) yield normal wings while non-complementing combinations (—) yield *rudimentary* wings.

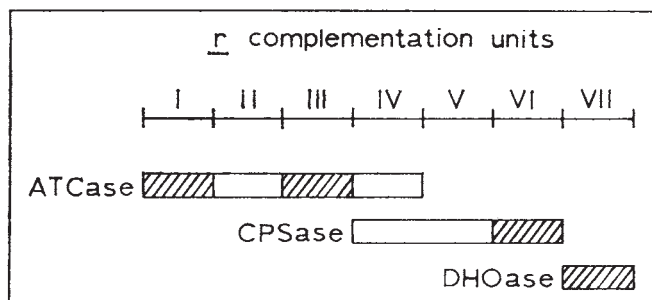


Fig. 1 Enzyme-specifying regions of the *rudimentary* complementation map. Complementation unit designations are from Carlson¹³. Horizontal bars indicate those units for which control of ATCase, CPSase, or DHOase levels is demonstrated (cross-hatched) or suspected (open). See text for discussion.

Allele complementation with respect to the wing phenes was verified for the *r* alleles used in this study. Table 1 shows that for alleles representing complementation groups I–IV, VI and VII, all possible heteroallelic combinations yielded normal winged flies, whereas the group I–VII allele failed to complement any other allele.

Previous studies have associated *r* complementation groups I–VII, V–VI and VI with CPSase deficiencies and groups I–VII, I and III with ATCase deficiencies^{16,18}. Table 2 corroborates the latter findings, showing ATCase activity to be greatly reduced by complementation group I–IV and group I–VII alleles. This enzymatic lesion is not found with alleles of group VI or group VII.

The third enzyme of the pyrimidine pathway, DHOase, is specified by complementation unit VII of the *r* locus. Table 2 shows that while flies homozygous for group I–VII or group VII alleles possessed much less DHOase than normal, activity was essentially normal in group I–IV and group VI individuals.

A map of the enzyme functions of the *r* locus is presented in Fig. 1. Complementation units I and III specify ATCase activity^{16,18}; unit VI directs CPSase activity¹⁸; unit VII is responsible for DHOase levels (Table 2). It is unlikely

that any of the three remaining *r* complementation units individually specify an unknown *r* function since their existence is only inferred and no *r* allele uniquely belongs to any of these units¹³. Unit II is presumably involved in ATCase expression since it lies between units I and III. Unit IV could affect ATCase and/or CPSase levels. Unit V probably directs CPSase along with unit VI since an allele belonging to complementation group V–VI has normal ATCase and lacks CPSase¹⁸. It must be pointed out that group IV–V alleles are known¹³; thus it is conceivable that these two units are jointly responsible for an unknown function of the *r* locus.

If an additional enzymatic function is specified by *r*, apparent biochemical and genetic correlations suggest later steps in the pyrimidine pathway. Accordingly, the fourth enzyme, dihydroorotate dehydrogenase (DHOase), was assayed in flies homozygous for a totally non-complementing allele. Table 3 shows that if anything, these flies had more DHOase activity than wild type flies. Thus, the ability to convert dihydroorotate to orotate is not diminished in *r* individuals.

It is important to know whether the *r* locus encodes the primary structures of the early pyrimidine enzymes or if it is a regulatory site, the product of which is essential for the synthesis, stability, or activation of these enzymes. Suggestive evidence regarding these possibilities is gleaned from the response of enzyme activity to changes in gene dose, thus Table 2 also presents enzyme values for flies heterozygous for *r* mutations. Clearly, alleles of *r* are codominant with respect to ATCase and DHOase activity; that is, females possessing one mutant and one wild type dose of the locus have enzyme levels intermediate between mutant and wild type homozygotes. Flies that possess three normal doses of the ATCase and DHOase regions of *r* (*r*^{39k}/*r*^{39k}/*Dp(1;4)r*⁺*f*⁺) have approximately 50% more ATCase and DHOase than flies with two doses of these regions, which suggests that the availability of *r* gene copies is rate-limiting in the expression of these enzymes. Since strict proportionality between gene dose and enzyme activity might be an exclusive property of enzyme structural genes^{22,23}, we

Table 2 ATCase and DHOase activities in females bearing various *r* alleles

Complementation group	Genotype	ATCase	DHOase	<i>n</i>
Wild type homozygote	<i>r</i> ⁺ / <i>r</i> ⁺	76 ± 15	26 ± 4	4
I-IV Homozygote	<i>g</i> ² <i>r</i> ^{59k23} <i>f</i> / <i>g</i> ² <i>r</i> ^{59k23} <i>f</i>	4 ± 1	17 ± 4	3
Heterozygote	<i>g</i> ² <i>r</i> ^{59k23} <i>f</i> / <i>Ins(1) sc</i> ⁵¹ + <i>d1</i> –49, <i>v</i> <i>r</i> ⁺	42 ± 8	17 ± 1	3
VI Homozygote	<i>g</i> ² <i>r</i> ^{39k} <i>f</i> / <i>g</i> ² <i>r</i> ^{39k} <i>f</i>	83 ± 25	21 ± 6	3
Heterozygote (aneuploid)	<i>g</i> ² <i>r</i> ^{39k} <i>f</i> / <i>g</i> ² <i>r</i> ^{39k} <i>f</i> / <i>Dp(1;4) r</i> ⁺ <i>f</i> ⁺	131 ± 12	33 ± 2	2
VII Homozygote	<i>r</i> ³⁷¹⁰ / <i>r</i> ³⁷¹⁰	89 ± 21	4 ± 2	5
Heterozygote	<i>r</i> ³⁷¹⁰ / <i>In(1) ClB</i> , <i>v</i> <i>r</i> ⁺ <i>B</i>	92 ± 23	14 ± 2	5
I-VII Homozygote	<i>r</i> ^{60d1} <i>f</i> / <i>r</i> ^{60d1} <i>f</i>	6 ± 1	4 ± 1	4
Heterozygote	<i>r</i> ^{60d1} <i>f</i> / <i>In(1) FM6</i> , <i>y</i> <i>w</i> <i>dm</i> <i>r</i> ⁺	41 ± 6	12 ± 2	4

Complementation group designations are after Carlson¹³ and all genetic markers and chromosomal rearrangements are described in detail elsewhere^{13,10}. Wild type females are from an Oregon-R strain. Flies were cultured at 25 °C on standard cornmeal-molasses-agar medium that was supplemented with 1% RNA to promote viability of *r* genotypes. Adult females 2–4 d of age were frozen at –20 °C for 1–4 d before assay. Samples were homogenized with glass tissue grinders at a concentration of 250 mg flies per ml of cold 50 mM potassium phosphate buffer (pH 7.2), 250 mM in sucrose. Homogenates were cleared by centrifugation for one hour at 20,000g and the supernatant fractions were desalted on small Bio-Gel P-2 columns equilibrated with buffer less sucrose. ATCase activity was determined by incubating a 500 µl mixture containing 200 mM Tris-HCl (pH 9.2), 20 mM potassium L-aspartate, 15 mM repurified dilithium carbamyl phosphate¹⁹, and 200 µl desalted extract. DHOase activity was measured in a 500 µl mixture containing 200 mM Tris-HCl (pH 8.0), 2 mM 4,5-dihydro-L-ornithine, and 300 µl desalted extract. Mixtures were incubated one hour at 30 °C and the carbamylaspartate formed was determined by the method of Gerhart and Pardee¹⁹. Values are derived from the difference between samples and blank reaction mixtures from which substrate was omitted. Activities are expressed as mean nmol carbamylaspartate formed per h per mg protein ± s.e. Protein was determined by the method of Lowry *et al.*²⁰. *n* is the number of separate samples assayed.

Table 3 DHodehase levels in wild-type and *r* females

Genotype	DHodehase activity
r^+/r^+ (Wild type)	16.0 ± 1.3
$r^{60d1} f/r^{60d1} f$ (Complementation group I-VII)	24.6 ± 0.8

Females from an Oregon-R wild type strain and from a homozygous strain of r^{60d1} were reared and homogenised as described in Table 2 except that they were not frozen before homogenisation. Homogenates were cleared of large cuticular debris by filtration through several layers of cotton gauze and glasswool. DHodehase activity was estimated by incubating 10 μ l filtered homogenate in a total volume of 50 μ l including 200 mM Tris-HCl (pH 8.0) and 2 mM 4,5-dihydro-DL- α -orotate (6- 14 C, New England Nuclear) (2.8 μ Ci μ mol $^{-1}$). This mixture was incubated at 30 °C for 40 min. The reaction was stopped by adding 10 μ l 10 N formic acid and the samples were frozen until chromatography. After centrifugation at 10,000g for 15 min to remove denatured protein, 5 μ l sample and 5 μ l of a carrier mixture (0.125 μ mol each of N-carbamyl-DL-aspartate, 4,5-dihydro-DL- α -orotate, and L-orotate) were mixed and spotted on to sheets of DEAE-cellulose paper (Whatman, DE-81). The sheets were developed for 3 h at room temperature using a formic acid-methanol-water solvent system²¹. With this method orotic acid ($R_f = 0.45$) is cleanly separated from dihydroorotate ($R_f = 0.70$). Carbamylaspartate, which is generated by endogenous DHodehase activity, chromatographs at $R_f = 0.80$. 14 C-orotate is not further converted to orotidylate or uridylate because of lack of phosphoribosylpyrophosphate in these reaction mixtures. The orotic acid zone of each chromatogram was located by its fluorescence under ultraviolet light, cut out, and 14 C-orotate was quantitated in a liquid scintillation system. Activity is expressed as mean nmol orotate formed per 40 min per mg protein $\pm$ s.e. Each mean is derived from determinations on three different samples. Protein was determined by the method of Lowry *et al.*¹⁰.

believe that the *r* locus contains the structural genes of the first three enzymes in pyrimidine biosynthesis.

In addition to providing genetic insight into the early steps of the pyrimidine pathway in animals, the presence of multiple cistrons within this locus in *Drosophila* has several implications for gene regulation and genome structure of higher eukaryotes. Most non-complementing *r* alleles are clustered near the ATCase end of the fine structure map^{12,13}. Such polar mutants could define a *cis*-dominant regulatory element or may represent frameshift and non-sense mutations within a cotranscribed and translated multicistronic region. Thus, polycistronic mRNA could mediate the synthesis of the early pyrimidine enzymes in animals as well as in fungi¹⁻⁷. Secondly the estimated size of the *r* locus, 24,000 base pairs of DNA, agrees closely with the average DNA content of a *Drosophila* chromomere, suggesting that this multicistronic locus resides within a single chromomere²⁴. If so, *rudimentary*, and other "complex" loci are exceptions to a "one gene-one band" hypothesis²⁵, and it becomes imperative to determine the precise cytogenetic location of *r*. Lastly, a variety of genetic tests (frequency of gene conversion, intralocus map distance, complementation) indicate that the *r* locus is but one of a group of "complex" loci (for example, *white*, *Notch*, *dumpy*, *lozenge* and *miniature*)²⁴ that have a multicistronic nature.

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¹ Jones, M. E., *Curr. Top. cell. Regul.*, **6**, 227-265 (1972).

² Shoaf, W. T., and Jones, M., *Biochemistry*, **12**, 4039-4051 (1973).

³ Davis, R. H., *Science*, **178**, 835-840 (1972).

⁴ Denis-Duphil, M., and Lacroute, F., *Molec. gen. Genet.*, **112**, 354-364 (1971).

⁵ Gans, M., and Masson, M., *Molec. gen. Genet.*, **105**, 164-181 (1969).

⁶ Davis, R. H., *Proc. natn. Acad. Sci. U.S.A.*, **46**, 677-682 (1960).

⁷ Radford, A., *Molec. gen. Genet.*, **104**, 288-294 (1969).

⁸ Caroline, D. F., *J. Bact.*, **100**, 1371-1377 (1969).

⁹ Morgan, T. H., *Am. Nat.*, **49**, 240-250 (1915).

¹⁰ Lindsley, D. L., and Grell, E. H., *Carnegie Inst. Wash. Publ.* No. 627 (1968).

¹¹ Fahmy, O. G., and Fahmy, M. J., *Nature*, **184**, 1927-1929 (1959).

¹² Green, M. M., *Genetica*, **34**, 242-253 (1963).

¹³ Carlson, P. S., *Genet. Res., Camb.*, **17**, 53-81 (1971).

¹⁴ Nørby, S., *Hereditas*, **66**, 205-214 (1970).

¹⁵ Falk, D., and Nash, D., *Genetics*, **76**, 755-766 (1974).

¹⁶ Nørby, S., *Hereditas*, **73**, 11-16 (1973).

¹⁷ Nørby, S., *Hereditas*, **73**, 140 (1974).

¹⁸ Jarry, B., and Falk, D., *Molec. gen. Genet.*, **135**, 113-122 (1975).

¹⁹ Gerhart, J. C., and Pardee, A. B., *J. biol. Chem.*, **237**, 891-896 (1962).

²⁰ Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. biol. Chem.*, **193**, 265-275 (1951).

²¹ Herrman, E. C., Dunn, J. H., and Schmidt, R. R., *Analyt. Biochem.*, **53**, 478-483 (1973).

²² O'Brien, S. J., and Gethmann, R. C., *Genetics*, **75**, 155-167 (1973).

²³ Rawls, J. M., and Lucchesi, J. C., *Genet. Res., Camb.*, **24**, 59-72 (1974).

²⁴ Fristrom, J. W., and Yund, M. A., *Crit. Rev. Biochem.*, **1**, 537-570 (1973).

²⁵ Lefevre, G., *A. Rev. Genet.*, **8**, 51-62 (1974).

Radiation-induced DNA strand breaks in *E. coli* measured within a fraction of a second

The yield of radiation-induced strand breaks in DNA is in normal conditions, larger in oxygenated cells than in anoxic cells. This may reflect either radiochemical reactions or preferential repair of breaks formed in anoxia. It is possible to distinguish between these possibilities by experiments in which irradiation and cell lysis are completed within a time period sufficiently short to minimise repair. We have measured in oxic and anoxic cells the yield of strand breakage on bacteriophage λ DNA superinfecting lysogenic bacteria¹ and now on chromosomal DNA in experiments where irradiation and lysis are carried out within a fraction of a second.

Figure 1 shows the results obtained when bacteria labelled with 3 H-thymidine were irradiated, rapidly lysed and then sedimented in alkaline sucrose gradients with non-irradiated 32 P λ DNA as a marker. The broad distribution of chromosomal DNA is as expected for a polydisperse collection of DNA molecules resulting from random strand breakage. After a dose of 12-13 krad in oxygen the peak of the 3 H-DNA sediments to the same position as does the 32 P λ marker DNA, the molecular weight of which is 15×10^6 . For randomly broken DNA, the peak of radioactivity in an alkaline sucrose gradient occurs at a molecular weight that is 1.62 times greater than the average molecular weight². Thus, the average molecular weight of the chromosomal DNA after a dose of 12-13 krad is 9.3×10^6 . From these values, the yield of breaks in chromosomal DNA is calculated to be 8.3 to 8.9×10^{-12} breaks per dalton per rad. In nitrogen anoxia, about 35 krad are needed to make the peak of the 3 H-DNA cosediment with the 32 P λ marker DNA. Thus, in nitrogen the yield of breaks in chromosomal DNA decreases about threefold to 3.0×10^{-12} breaks per dalton per rad.

An increase in strand breaks in anoxic bacteria was found in *Micrococcus radiodurans* irradiated at high pH in the presence of EDTA (ref. 3) and in *Escherichia coli* subjected to heat treatment, osmotic cold shock, N-ethylmaleimide or quinacrine⁴. The authors^{3,4} assumed that these treatments inhibited strand joining and concluded that the difference in radiation-induced strand breakage observed in oxygenated and anoxic cells is not caused by a difference in the initial number of radiochemical strand breaks, but by a novel and rapid repair mechanism that preferentially rejoins breaks produced in anoxia. If the oxygen effect observed for strand breakage in chromosomal DNA (Fig. 1) is caused by preferential repair, the repair process must have gone to completion within less than 200 ms in buffer at room temperature. From recent experiments⁵, some conclusions can be drawn about the probable extent of strand joining in the conditions used in these experiments. The authors⁵ estimated that the maximum number of single-strand breaks in an *E. coli* cell at 30 °C that could be rejoined per second is about 130. At 20 °C the activity of *E. coli* DNA ligase is only 0.57 of that

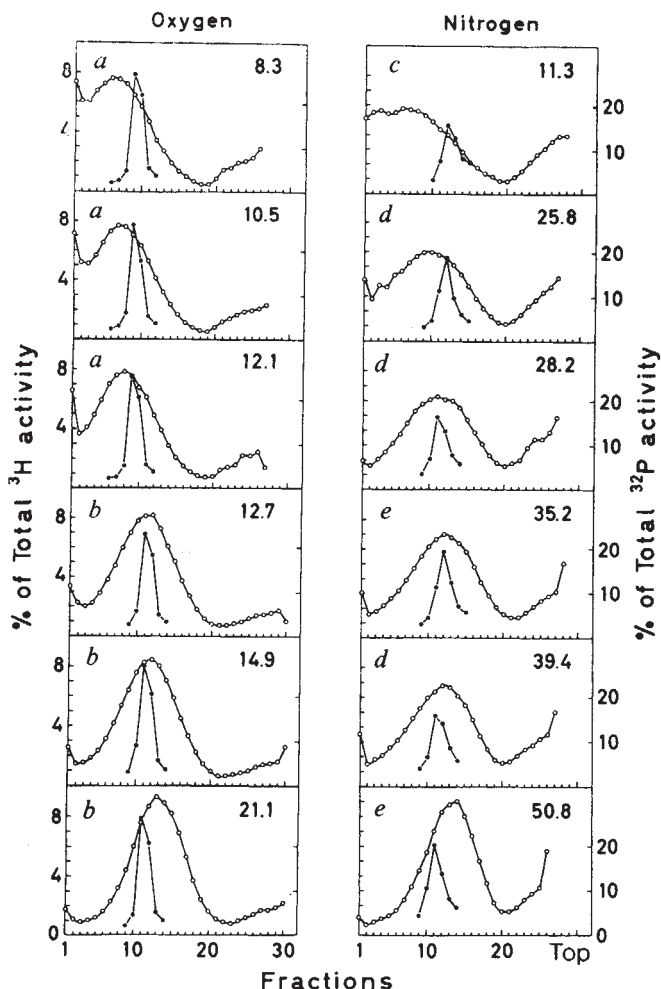


Fig. 1 Sedimentation profiles of chromosomal DNA after irradiation and lysis within a fraction of a second. Suspensions of *E. coli* K12 strain AB1886, ^3H -labelled in the DNA, in phosphate buffer (pH 6.8, room temperature) were equilibrated with oxygen or high purity nitrogen (<1 p.p.m. O_2). The cell suspensions were exposed to 4 MeV electrons from a linear accelerator with the doses indicated (krad), and rapidly transferred to alkaline detergent¹. Times required to complete the experiments were between 75 and 95 ms for the oxygenated series and between 70 and 330 ms for the anoxic series, and include 45 ms needed for transfer of the cells to the detergent. The lysates were sedimented in 5–20% alkaline sucrose gradients together with non-irradiated ^{32}P -labelled phage λ DNA (Beckman SW50.1 rotor, 40,000 r.p.m., 20 °C, 2 h). Ten drop fractions were collected from the bottom of the tubes directly into scintillation liquid and the ^3H (○) and ^{32}P (●) activities measured for each fraction. Identical letters indicate that the profiles were obtained from the same run.

at 30 °C (ref. 6). Thus, at room temperature the maximum estimated number of strand-joining events would be less than 15 per cell in 200 ms. This can be compared with the number of strand breaks present in the cell after exposure to 4 MeV electrons. For an experiment of 200 ms duration about 27 krad are delivered. Assuming that in our growth conditions there is 15×10^{-15} g DNA per cell⁷, there will after 27 krad be about 700 breaks per anoxic cell, and about 2,100 breaks per oxic cell. Thus, if rapid strand joining is to account for the difference of 1,400 breaks in oxic and anoxic cells, the strand-joining capacity must be at least 90 times greater than that estimated by Modrich and Lehman⁵.

In separate experiments, the time needed for joining λ DNA molecules broken by pulsed electron radiation was measured⁸. In growth conditions, no strand joining was observed during the first 3 s of incubation. In the presence of oxygen most strand breaks were rejoined during 40 s incubation, whereas the rejoining was even slower in nitrogen anoxia.

Table 2 Effect of high pH on cellular sulphydryls

Treatment*	Sulphydryls in cell free supernatant (mM)
Phosphate buffer, pH 6.8	0.05
Veronal buffer, pH 8.6	0.57
Veronal buffer, pH 8.6 + 0.02 M EDTA	0.59
Tris buffer, pH 8.6	1.22
Tris buffer, pH 8.6 + 0.02 M EDTA	0.91

*Cells of *E. coli* K12 from a 100 ml log-phase suspension were collected by centrifugation and washed in phosphate buffer at pH 6.8. The cells were resuspended in 0.3 ml of the buffer as indicated, kept for 10 min at room temperature and the concentration of sulphydryls measured in the supernatant.

As we have found no experimental evidence for the existence of a very rapid strand break repair mechanism in anoxic cells, we do not believe that the explanation of the oxygen effect on observed strand breaks involves repair. The possibility that the experiments demonstrating changes in the oxygen effect by chemical and physical treatment of cells before irradiation^{3,4} may reflect changes in the concentration of sulphydryl compounds in the cell, was tested in separate experiments. Table 1 shows that after a heat treatment of 52 °C for 10 min⁴ more than 50% of the acid-soluble sulphydryls in the cells have leaked out into the suspending medium. This is paralleled by an increase in the number of radiation-induced DNA strand breaks in hypoxic and anoxic conditions. Thus, heat-shocked cells are sensitised twofold by a small contaminating concentration of oxygen (125 p.p.m.). The same effect is observed in cells treated with the sulphydryl-binding agent N-ethylmaleimide⁹. A slightly milder heat treatment, 48 °C for 5 min, had neither significant effect on the leakage of sulphydryls out from the cells nor on the yields of radiation-induced DNA strand

Table 1 Effect of heat shock on cellular sulphydryls and on DNA strand breakage

Treatment*	Sulphydryls in cell free supernatant (mM)	1/e dose for DNA single-strand break†		
		O_2	0.0125% O_2	N_2
20 °C, 10 min	0.19	7.1	—	38.7
Heat shock: 52 °C, 10 min	0.74	5.0	18.2	12.5–35.0‡
Heat shock: 48 °C, 5 min	0.24	6.1	34.7	40.5

*Cells of *E. coli* K12 from a 100 ml log-phase suspension were collected by centrifugation, suspended in 0.3 ml phosphate buffer at pH 6.8 and subjected to temperatures as indicated. After treatment the concentration of sulphydryls was measured in the cell free supernatant by the sodium nitroprusside reaction¹¹. When the bacteria were suspended in 0.3 ml TCA at 0.3 M the concentration of sulphydryls in the supernatant was 1.38 mM.

†X-ray induced DNA single-strand breaks were measured in superinfecting, covalently closed circular phage λ DNA by sedimentation in alkaline sucrose¹.

‡X irradiation caused an exponential decrease in the number of intact circular DNA molecules, but the 1/e dose observed in individual experiments varied between the values shown. In the presence of $\text{Na}_2\text{S}_2\text{O}_4$ at 500 μM —a substance that combines quickly with oxygen—a 1/e dose of about 33 krad was repeatedly found.

breaks. Table 2 shows that a substantial amount of acid-soluble sulphhydryls leaks out of the cells when suspended in veronal buffer at pH 8.6 supplemented with EDTA at 0.02 M (ref. 3). This effect is not caused by the presence of EDTA as it is observed with veronal buffer alone. The effect is more likely a result of the high pH as a substantial leakage of sulphhydryls is also observed from cells suspended in Tris buffer at pH 8.6.

On this basis, we think that previous experiments^{3,4} showing diminished anoxic protection for single-strand breaks in the treated cells, have been misinterpreted and should not be attributed to the loss of a rapid enzymatic repair process. Instead, we suggest that the effect may be caused by the fall in the level of sulphhydryl compounds, which are known to act as hydrogen donors in radiochemical reactions and to contribute to anoxic radiobiological protection¹⁰.

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- ¹ Johansen, I., Brustad, T., and Rupp, W. D., *Proc. natn. Acad. Sci. U.S.A.*, **72**, 167-171 (1975).
- ² Rupp, W. D., and Howard-Flanders, P., *J. molec. Biol.*, **31**, 291-304 (1968).
- ³ Dean, C. T., Ormerod, M. G., Seriani, R. W., and Alexander, P., *Nature*, **222**, 1042-1044 (1969).
- ⁴ Town, C. D., Smith, K. C., and Kaplan, H. S., *Radiat. Res.*, **52**, 99-114 (1972).
- ⁵ Modrich, P., and Lehman, J. R., *J. biol. Chem.*, **248**, 7502-7511 (1973).
- ⁶ Olivera, B. M., and Lehman, J. R., *J. molec. Biol.*, **36**, 261-274 (1968).
- ⁷ Cooper, S., and Helmstetter, C. E., *J. molec. Biol.*, **31**, 519-540 (1968).
- ⁸ Boye, E., Johansen, I., and Brustad, T., *J. Bact.*, **119**, 522-526 (1974).
- ⁹ Johansen, I., Gulbrandsen, R., and Pettersen, R., *Radiat. Res.*, **58**, 384-397 (1974).
- ¹⁰ Adams, G. E., McNaughton, G. S., and Michael, B. D., *Trans. Faraday Soc.*, **64**, 902-910 (1968).
- ¹¹ Grunert, R. R., and Phillips, P. H., *Archs Biochem.*, **30**, 217-225 (1951).

Acquisition of tumour-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer

It has been suggested that the tumour-inducing capacity of *Agrobacterium* is determined by plasmid genes^{1,2}. The acquisition of virulence by non-pathogenic agrobacteria from pathogenic agrobacteria has been demonstrated by Kerr^{3,4}. We investigated whether this phenomenon could be the result of transfer of a plasmid. Besides providing further evidence for the hypothesis that plasmid genes carry the genetic information for the tumour-inducing ability of *Agrobacterium*, the system offers the possibility of studying the genetics and functions of the plasmid.

The donor and recipient strains of *Agrobacterium* must be well-characterised. Donor strain *A. rhizogenes* 223, used in a first set of experiments, has the typical biochemical properties of cluster 2 (ref. 5). It cannot produce 3-ketolactose, does not grow anaerobically on nitrate containing medium, grows slowly and is resistant to all of the seven different types of virulent phages of our typing set (see Table 1). The cured non-tumour-inducing receptor strain C58-C1 was derived from a single colony of *A. tumefaciens* C58 by growth at 37 °C, which resulted in both the loss of its plasmid and the ability to induce tumours². From C58-C1 a double mutant was isolated which showed resistance to 100 µg ml⁻¹ rifampicin and to 300 µg ml⁻¹ streptomycin. This C58-C1 *rif str* was repeatedly tested for plasmid and tumour-inducing ability and was always found to be negative for both. *A. tumefaciens* C58-C1 *rif str* belongs to the TT9 group of cluster 1 (ref. 5), can produce 3-ketolactose,

can grow anaerobically and shows, among our laboratory strains, a unique and typical sensitivity pattern to the virulent phages of our typing set (see Table 1). We crossed the oncogenic *A. rhizogenes* strain 223 with the non-oncogenic *A. tumefaciens* strain C58-C1 *rif str* on *Kalanchoë daigremontiana* as host plant. The experimental procedures were as described by Kerr⁴. Eight weeks after inoculation 15 out of 30 reisolated single colonies of the antibiotic-resistant recipient bacteria seemed to be oncogenic on 1-week-old sunflower seedlings. A pathogenic colony was then subjected to further extensive purification. The resulting bacteria showed the antibiotic resistance, positive 3-ketolactose test, anaerobic growth and the phage sensitivity pattern characteristic of the avirulent C58-C1 *rif str* strain used as recipient (Table 1). They were repeatedly found to induce crown gall tumours on pea seedlings, sunflower seedlings and *Kalanchoë daigremontiana*. Using ultracentrifugation^{1,2} and subsequent electron microscopy we demonstrated, in the purified newly oncogenic recipient bacterium, a large plasmid with a mean length of 58.0 µm ± 1.3 µm s.d., which lies in the range 61.1 µm ± 1.4 µm s.d. that we found for a sample of plasmid molecules isolated from the donor strain 223. *A. tumefaciens* C58-C1 *rif str* also acquired other characteristics that are known to be coded for by the plasmid, namely sensitivity to agrocins 84 and 396 (G. Engler, J.P.H., M. Holsters, M.V.M., R.A.S., and J.S., unpublished), the ability to degrade an arginine derivative nopaline (ref. 6 and G. M. Bomhoff, R.A.S., J.P.H., H. Kester, and J.S., unpublished) and the ability to exclude the virulent *Agrobacterium* phage AP1 (J.P.H., M.V.M., and J.S., unpublished). Strain C58-C1 *rif str* (TI223) differs from the parental oncogenic strain C58 in that it cannot be cured of its plasmid by growth at 37 °C, even after 75 generations, whereas a culture of the parental strain C58 is cured completely after 25 generations of growth at 37 °C. This property apparently depends on the nature of the plasmid present in the bacterium.

To confirm these results we performed a better controlled experiment on otherwise sterile pea seedlings in sterile test tubes containing agar medium. This cross involved the crown gall-inducing *Agrobacterium tumefaciens* strain Kerr 14 and the naturally occurring non-oncogenic *Agrobacterium radiobacter* S1005, made resistant against 100 µg ml⁻¹ rifampicin and 300 µg ml⁻¹ streptomycin. The pea seedlings were inoculated with S1005 *rif str* 12 d after inoculation with *A. tumefaciens* Kerr 14. As controls we used pea seedlings that were only inoculated with either Kerr 14 or S1005 *rif str*. Using sensitivity to agrocin 84 as a screening test we were able to pick up a single colony that was still resistant to 100 µg ml⁻¹ rifampicin and to 300 µg ml⁻¹ streptomycin, had the typical sensitivity pattern of *A. radiobacter* S1005 in a test with our typing set of seven different virulent *Agrobacterium* bacteriophages (Table 1), still produced the typical agrocin S1005, effective against *A. tumefaciens* B6-S3, but had become oncogenic as shown by tests on the pea seedlings⁷. By ultracentrifugation and subsequent electron microscopy this oncogenic *A. radiobacter* S1005 (TI Kerr 14) was shown to harbour a large plasmid. The strain had also become sensitive to agrocins 84 and 396, showed an efficient exclusion of phage AP1 and could degrade nopaline. On control pea seedlings inoculated with the recipient strain S1005 *rif str* no tumours were found. From uninoculated pea seedlings no bacteria could be isolated after 6 weeks. From tumours induced by *A. tumefaciens* Kerr 14 but not contaminated with S1005 *rif str* no bacteria could be isolated that were resistant to rifampicin and streptomycin.

These results show that the transfer of virulence as observed by Kerr, rests on a transfer of a large plasmid from the oncogenic strain to the avirulent recipient. By the acquisition of such a plasmid from a taxonomically distant strain, a strain that had been made non-oncogenic by curing of its plasmid as well as a naturally occurring non-oncogenic *A. radiobacter* strain could be rendered oncogenic. Crown gall tumour-inducing ability, ability to degrade nopaline, the exclusion of virulent phage AP1 and the sensitivity to agrocins 84 and 396 are all phenotypic properties coded for by one large plasmid. As tumour-inducing

Table 1 Properties of *Agrobacterium* strains used

Strain	Resistance to 100 µg ml ⁻¹ rifampicin	Resistance to 300 µg ml ⁻¹ streptomycin	Ability to produce 3-ketolactose ^{1,2}	Ability to grow anaerobically on nitrate-contain- ing medium ³	Sensitivity to virulent phages of typing set						
					GS1	GS2	GS3	GS5	GS6	GS18	AP1
223	—	—	—	—	—	—	—	—	—	—	—
C58-C1	+	+	+	+	—	+	—	+	+	—	+
C58-C1 (TI 223)	+	+	+	+	—	+	—	+	+	—	+
Kerr 14	—	—	+	+	+	—	+	—	+	+	+
S1005 rif ^{str}	+	+	+	+	+	+	—	—	+	—	+
S1005 rif ^{str} (TI Kerr 14)	+	+	+	+	+	+	—	—	+	—	+

Strain	Ability to degrade nopaline	Exclusion of phage AP1	Sensitivity to agrocin 84 and 396	Oncogenicity	Presence of a large plasmid	
223	+	—	+	+		+
C58-C1	—	—	—	—		—
C58-C1 (TI 223)	+	+	+	+		+
Kerr 14	+	+	+	+		+
S1005 rif ^{str}	—	—	—	—		—
S1005 rif ^{str} (TI Kerr 14)	+	+	+	+		+

plasmids from two distantly related oncogenic *agrobacteria* conferred these same properties to their new host, some of these properties may be related to the process of tumour induction. It has been suggested⁸ that the receptor sites for agrocin 84 could be part of a specific molecular configuration on the bacterial cell surface necessary for interaction with a proposed specific receptor site on the plant cell wall⁹⁻¹¹. The correlation between oncogenicity and agrocin 84 sensitivity could however, be simply a result of genetic linkage. The ability to degrade arginine derivatives could be part of a biochemical pathway which, when operating in a plant cell, leads to the production of these amino acids, which in itself could be important in tumour development, or, alternatively or additionally, could sustain further growth of the infecting pathogenic bacteria.

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¹ Zaenen, I., Van Larebeke, N., Teuchy, H., van Montagu, M., and Schell, J. *J. molec. Biol.*, **86**, 109-127 (1974).

² Van Larebeke, N., et al., *Nature*, **222**, 169-170 (1974).

³ Kerr, A., *Nature*, **223**, 1175-1176 (1969).

⁴ Kerr, A., *Physiol. Pl. Pathol.*, **1**, 241-246 (1971).

⁵ Kersters, K., de Ley, J., Sneath, P. H., and Sackin, M., *J. gen. Microbiol.*, **78**, 227-239 (1973).

⁶ Bomhoff, G. H., thesis, Univ. Leiden (1974).

⁷ Manigault, P., Kurkdjian, A., *C.r. hebdo. Séanc., Acad. Sci. Paris., Sér. D.*, **264**, 2304-2306 (1967).

⁸ Roberts, W. P., and Kerr, A., *Physiol. Pl. Pathol.*, **4**, 81-91 (1974).

⁹ Lippincott, B. B., and Lippincott, J. A., *J. Bact.*, **97**, 620-628 (1969).

¹⁰ Schilperoort, R. A., thesis, Univ. Leiden (1969).

¹¹ Beiderbeck, R., *Z. für Naturforschung*, **28c**, 198 (1973).

¹² Bernaerts, M. J., and de Ley, J., *Nature*, **197**, 406-407 (1963).

is illustrated most vividly by the serine proteinases¹ and by the haemoglobin-myoglobin family of haem proteins². The most surprising aspect of the very close similarity in conformations within these protein families is the very large variation in primary structure which is compatible with a given protein conformation; for example, only 3 of the 140 to 150 amino acid residues are invariant in the sequences which give the myoglobin-like conformation.

Examination of this aspect of protein structure in a small, compact protein, with fewer degrees of freedom in its amino acid sequence and its folded structure, such as the bovine pancreatic trypsin inhibitor (BPTI), is useful when considering the relationship between amino acid sequence and protein conformation. The tertiary structure of BPTI has been determined to very high precision by Huber³ and his colleagues⁴. The amino acid sequences of six other proteins, from sources as diverse as cows, snails, snake toxins and turtle eggs, are homologous with BPTI (Fig. 1). All are proteinase inhibitors. The conformations of none of the homologous proteins have been determined, but consideration of the amino acid sequences in terms of the known tertiary structure of BPTI leaves little doubt that all have very similar conformations.

The numbering of the amino acid residues throughout is that of BPTI, and only residues 1-55 are considered to be of significance. There are no deletions or additions of residues within the primary structure. The BPTI conformation is remarkably compact and rigid, and it is unlikely that any such alterations could be accommodated.

The six cysteine residues, which form three disulphide bonds in BPTI, are invariant. The pairings of cysteine residues in disulphide bonds have been shown to be the same in the bovine colostrum inhibitor¹¹ and the Russell's viper inhibitor⁸, as would be required for the BPTI conformation. Two of the disulphide bonds of BPTI (5-55 and 30-51) are required for maintenance of the native conformation, whereas the third seems to stabilise the protein for its role as a proteinase inhibitor¹².

BPTI has only a small proportion of interior residues, because of its small size, but the aromatic residues 22, 23, 33, 35 and 45, the hydrophobic residues 4 and 21, and Asn-43, the side chain of which participates in three apparently crucial hydrogen bonds in the centre of the molecule, are clearly the most important. All these residues are highly conserved in all the homologous proteins.

Most of the amino acid side chains are on the surface of the molecule and vary widely among the seven proteins. In no case, however, does one of these residues ha-

Homology of protein structures: proteinase inhibitors

HOMOLOGY of amino acid sequences is often accompanied by close similarity in the protein tertiary structures. This

[illegible]

Fig. 1 Amino acid sequences of seven homologous proteinase inhibitors: BPTI (ref. 5); bovine colostrum trypsin inhibitor⁶; turtle egg white inhibitor⁷; snail inhibitor K (ref. 8); Russell's viper venom inhibitor⁹; toxins I and K of black mamba venom¹⁰.

large hydrophobic side chain extending into the solvent, which would contradict one of the most obvious rules of protein structure. In certain positions, such as residue 18, non-polar side chains extend to the surface, but they also participate in seemingly important interactions within the structure. Black mamba toxin I has Tyr at residue 15, but this residue is of prime importance for interaction with proteinases.

Glycine residues may attain conformational parameters which are inaccessible for stereochemical reasons to all other amino acid residues; the Gly residues at positions 12, 28, and 37 of BPTI have conformational angles outside the permissible range⁴. In all cases, Gly is invariant at positions 12 and 37, and the model of BPTI further shows that these residues are interior, with no room for a side chain. Residue 28 has been changed, however, to other amino acids in most of the other homologous proteins; these side chains would be on the surface of the molecules, but the conformational angles of this residue in these proteins must be somewhat different from those in BPTI.

Proline residues markedly restrict the conformational angles attainable by the peptide chain (φ must be in the region of -80°)¹³. Replacement of Pro by any other amino acid is not affected by such restrictions, but to substitute Pro for any other amino acid without altering the backbone conformation would only be possible if the conformation were compatible with a Pro residue. Such substitutions have occurred in the homologous proteins at positions 11, 19, 25 and 32. The values of φ for these four non-Pro residues in BPTI (refs 3 and 4) range from -64° to -86° , which are within the range observed with the four Pro residues of BPTI, -59° to -89° . Therefore, the additional Pro residues of the other proteins could be incorporated readily with the BPTI structure.

The amino acid sequences of the six homologous proteins shown in Fig. 1 each seem to be compatible in virtually all respects with the three-dimensional folded conformation of BPTI. The consistency is so striking as to leave

little doubt that the native proteins have the BPTI conformation.

The diverse phylogeny of the species in which the seven proteins of Fig. 1 are found, with the usual assumption of evolutionary divergence of structural genes from an ancestor common to all the species, makes it unlikely that the divergence between the primary structures was limited by the rate of mutation. Rather, the residues common to all must have been conserved by natural selection to maintain the native structure, and possibly the function, of the proteins. The physiological functions of the proteins are not known, but, as all are proteinase inhibitors, it is likely that all have functions related to this property. Detailed studies of the proteinase inhibitor properties of BPTI (ref. 14) have indicated that only a few residues in the vicinity of residue 15 participate directly in binding to proteinases. Numerous alterations of the molecule away from this region do not affect the inhibitor activity, as long as the conformation of the molecule is not affected. Therefore, the striking conservation of certain residues in the sequences of Fig. 1 pinpoints those residues crucial for maintaining the folded conformation.

Assumption of any other scheme for the evolutionary origin of the structural genes of the seven proteins (including convergent evolution), using known mechanisms of gene transfer in such organisms, would also require that the striking similarities of certain residues result from requirements of protein conformation.

The six proteins homologous to BPTI presumably are also able to fold into the native conformation on a normal time scale, as does BPTI (ref. 15). These homologous proteins should then be of use in elucidating, both theoretically¹⁶ and experimentally¹⁵, the basis of this remarkable feat.

The apparent concomitance of homology at the levels of both amino acid sequence and three-dimensional conformation requires that a possible primary structure homology also be tested in terms of tertiary structure when possible. The primary structure of an inhibitor of bromelain has

recently been suggested¹⁷ to be homologous to the seven proteins of Fig. 1. Consideration of the primary structure of the bromelain inhibitor in terms of the BPTI conformation, however, makes it seem very unlikely that the bromelain inhibitor could have a similar conformation.

The Cys residues of the bromelain inhibitor do not align exactly with those of BPTI, implying insertion and deletion of residues. The bromelain inhibitor has five disulphide bonds, but the four additional Cys residues would not be in relative positions in the BPTI conformation such that disulphide bonds could be formed. The retention of aromatic and hydrophobic residues at positions 4, 22, 23 and 35 in the homologous proteins is not present in the bromelain inhibitor. Large hydrophobic side chains at positions 17, 25, and 28 of the bromelain inhibitor would probably extend into the solvent. The presence of residues other than Gly at positions 12 and 37, and of Pro at position 15, of the bromelain inhibitor would not be permitted with the BPTI conformation. It thus seems very unlikely that the bromelain inhibitor is structurally homologous with BPTI and the six other proteins of Fig. 1.

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- 1 Hartley, B. S., *Phil. Trans. R. Soc.*, **B257**, 77-87 (1970).
- 2 Vainshtein, B. K. *et al.*, *Nature*, **254**, 163-164 (1975).
- 3 Huber, R., Kukla, D., Ruhmann, A., Epp, O., and Formanek, H., *Naturwissenschaften*, **57**, 389-392 (1970).
- 4 Deisenhofer, J., and Steigemann, W., *Acta Cryst.*, **B31**, 238-250 (1975).
- 5 Kassell, B., and Laskowski, M., Sr, *Biochem. biophys. Res. Commun.*, **20**, 463-468 (1965).
- 6 Cechová, D., Svestková, V., Keil, B., and Sorm, F., *FEBS Lett.*, **4**, 155-156 (1969).
- 7 Laskowski, M., Jr, Kato, I., Leary, T. R., Schrodde, J., and Sealock, R. W., in *Proteinase Inhibitors* (edit. by Fritz, H., Tschesche, H., Greene, L. J., and Truscheit, E.), 597-611 (Springer, Berlin, 1974).
- 8 Dietl, T., and Tschesche, H., in *Proteinase Inhibitors* (edit. by Fritz, H., Tschesche, H., Greene, L. J., and Truscheit, E.), 254-264 (Springer, Berlin, 1974).
- 9 Takahashi, H., Iwanaga, S., Kitagawa, T., Hokama, Y., and Suzuki, T., in *Proteinase Inhibitors* (edit. by Fritz, H., Tschesche, H., Greene, L. J., and Truscheit, E.), 265-276 (Springer, Berlin, 1974).
- 10 Strydom, D. J., *Nature new Biol.*, **243**, 88-89 (1973).
- 11 Cechová, D., and Ber, E., *Coll. Czech. chem. Commun.*, **39**, 680-688 (1974).
- 12 Wilson, L. F., and Laskowski, M., Sr, *J. biol. Chem.*, **246**, 3555-3561 (1971).
- 13 Ramachandran, G. N., and Sasisekharan, V., *Adv. Protein Chem.*, **23**, 283-437 (1968).
- 14 Rühlmann, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R., *J. molec. Biol.*, **77**, 417-436 (1973).
- 15 Creighton, T. E., *J. molec. Biol.*, **87**, 563-577, 579-602, 603-624 (1974); *J. molec. Biol.* (in the press).
- 16 Levitt, M., and Warshel, A., *Nature*, **253**, 694-698 (1975).
- 17 Reddy, M. N., Keim, P. S., Heinrichson, R. L., and Kezdy, F. J., *J. biol. Chem.*, **250**, 1741-1750 (1975).

Immune exclusion is a function of IgA

PREVENTION of entry of antigen into the systemic circulation through mucous membranes depends partly on the physical integrity of the mucous membranes and the mucous layer and, no doubt, on other nonspecific mechanisms. These systems are, however, not completely effective and there is also a specific acquired capacity for immune exclusion of antigen. For example, everted gut sacs from rats previously fed with an antigen show specific reduced transmission of the antigen, as compared with sacs prepared from animals not fed in this way¹. Passive transfer *in vivo*, using cell free homogenates of mucosae from antigen-fed animals has also been reported but the mechanism was not identified². One possibility is IgA antibody, the principal immunoglobulin of mucous secretions³, and this is supported by the demonstration of circulating antibody to food proteins in IgA deficient subjects⁴, and the association of allergic disease with IgA deficiency, either permanent⁵ or transient⁶.

The necessary passive transfer experiments, using pure IgA from the serum or secretions of an immunised animal are difficult. The problems can be avoided by using an IgA myeloma protein from an unimmunised animal. The mouse tumour MOPC-315 secretes an IgA myeloma protein which binds to

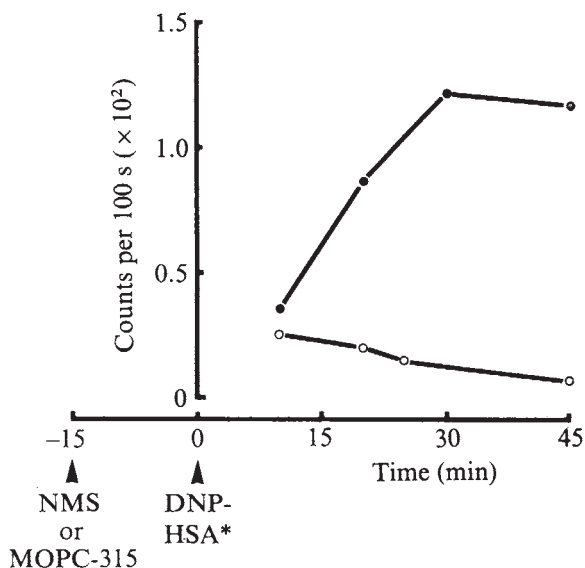


Fig. 1 Radioactivity in the serum of two rats injected into the trachea with ¹²⁵I-labelled DNP-HSA (DNP-HSA*) at time 0. Fifteen minutes previously the rats had received (also by intratracheal injection) either mouse serum containing MOPC-315 IgA myeloma protein (○) or normal mouse serum (●).

dinitrophenol (DNP) with affinity well into the antibody range⁷ at a concentration in serum of 15-20 mg ml⁻¹ (ref. 8). We have demonstrated that serum from unimmunised mice, containing this IgA protein, prevents the absorption through the rat respiratory tract of ¹²⁵I-labelled human serum albumin to which DNP is conjugated (DNP-¹²⁵I-HSA) but not ¹²⁵I-HSA.

Approximately 8 mm³ of MOPC-315 tumour were injected into each of 24 BALB/c mice, which were exsanguinated 21 d later. Their sera were pooled, and the IgA concentration found to be approximately 10 times that of normal mouse serum IgA (single radial diffusion using a rabbit anti mouse IgA). HSA (Kabi) was conjugated with DNP by a modification of Porter's method⁹, to achieve an average of 4 DNP groups per HSA molecule. DNP-HSA and HSA were labelled with ¹²⁵I by a chloramine T method¹⁰.

A range of concentrations of MOPC 315 serum (Neat—1:32,000 dilution) was reacted with DNP-¹²⁵I-HSA (0.25 µg) at 37 °C for 4 h and at 4 °C for 16 h. The mixtures were centrifuged, the supernatants discarded and residual radioactivity counted. Maximum 'counts bound' were obtained with a 1:4,000 dilution of the pooled serum and this was taken as equivalence for this system.

Wistar rats (200-400 g) were anaesthetised with avertin, the trachea exposed and 100 µl of either normal mouse serum or MOPC-315 serum injected into it. After 15 min 6.25 µg DNP-¹²⁵I-HSA (that is 250-fold less antigen than equivalent for the antigen binding properties of the myeloma serum) were injected into the trachea. The rats were then bled by cardiac puncture at timed intervals and the radioactivity in the serum counted in a Wallac counter.

The time sequence of radioactivity in the serum of a rat which received MOPC 315 serum and another which received normal mouse serum (NMS) are shown in Fig. 1. The rat receiving NMS showed increasing radioactivity, reaching a peak at 30 min, whereas the rat receiving MOPC 315 serum showed a low falling activity only. Similar sequences were found in 6 other rats, three in each group. A clear distinction was shown by 20 min, and anaesthesia for longer than this presented problems, so further work was done at this time. The distribution of radioactivity in the rats was observed by scanning with a Manual Gamma probe, connected to a rate-meter (Nuclear Enterprises Ltd). In both experimental and control groups most of the radioactivity remained in the thorax.

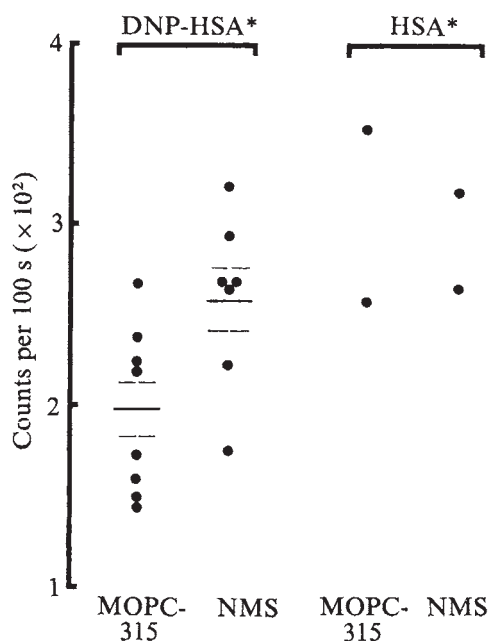


Fig. 2 Serum radioactivity in rats 20 min after intratracheal injections of either ^{125}I -labelled DNP-HSA (DNP-HSA*) or ^{125}I -labelled HSA (HSA*). They had received either mouse serum containing MOPC-315 IgA myeloma protein or normal mouse serum (NMS) into the trachea 15 min before. Mean $\pm$ s.e.m. are shown.

In a second experiment, rats were bled only at 20 min. The radioactivity in the serum was rather higher in both groups than in the initial experiments but it was significantly less ($t = 2.54$; $P < 0.025$) in the group receiving MOPC 315 serum than in that receiving NMS (Fig. 2). There was no such obvious difference in the serum radioactivity in four rats receiving ^{125}I -HSA instead of DNP- ^{125}I -HSA, after either MOPC-315 or normal mouse serum.

These experiments show that an IgA myeloma protein, injected into the rat respiratory tract, can specifically prevent entry into the blood of antigen for which it has binding affinity. This strengthens the view that IgA may be responsible for this process in both the respiratory tract, in which this is the first evidence of specific immune exclusion, as well, presumably, as in the gut where such a phenomenon has already been demonstrated² without the mechanism being established. The evidence that this mechanism is defective in IgA deficient individuals, namely their antibodies to food proteins⁴ and allergy^{5,6,11}, supports the view that IgA is important for this function, but immunodeficiency is often complex and other mechanisms might have been defective in such patients. Limited experiments with IgG rabbit anti-HSA antibody suggest that IgG may also be effective in our system. Walker and his colleagues¹² detected IgG1 anti-BSA antibody in extracts of intestinal mucosa and in intestinal secretions of rats immunised with BSA, so the IgG class may also be important, but the predominance of IgA in the secretions suggests that this is the main mechanism.

The use of the IgA myeloma protein avoids all the usual problems of establishing immunoglobulin class specificity of the observed biological effect, since the animals were not immunised. The negative controls using the DNP- ^{125}I -HSA with normal mouse serum and using ^{125}I -HSA with both MOPC 315 serum and NMS establish that it was an effect of IgA and exclude the possibility of nonspecific binding to HSA, a property of many IgA myeloma proteins¹³.

The observed antigen exclusion in our experiments was an effect of IgA which probably lacked secretory component. It is likely that normal secretory IgA antibody, with its reported resistance to proteolysis¹⁴, would be much more efficient. It is also possible, however, that the effect was due to polymerised IgA associated with secretory component derived from

secretions of the recipient rats. In either case we have shown that IgA can produce this effect, even though the artificial system used may be suboptimal.

It is attractive to suggest that in the gut the complexes of IgA antibody with antigen are retained in the lumen till the antigen is digested. In the respiratory tract they may be swept up by ciliary action, swallowed and similarly digested, although most of the antigen remained in the lungs in our acute experiments. If the adherence of IgA to mucous membranes¹⁵ is important in this system, it is possible that macrophages would be attracted by the chemotactic effect of complement, activated through the alternative pathway¹⁶.

If the function of immune exclusion were defective in an individual capable of eliciting IgE, IgG or cell mediated responses to the antigen, local allergic lung or gut damage or generalised soluble complex disease could occur. Even a transient defect such as in presymptomatic atopic infants⁶ could lead to persistent disease, since, once a population of sensitised cells is established, very little subsequent antigen entry would be required to maintain the process. Defects of the system we have described may, therefore, be the basis of many common diseases.

We thank Mr B. Gregory for help with the animals, Dr R. M. E. Parkhouse for a tumour bearing mouse, and the Chest and Heart Association for financial support.

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- Walker, W. A., Isselbacher, K. J., and Bloch, K. J., *Science*, **177**, 608 (1972).
- André, C., Lambert, R., Bazin, H., and Heremans, J. F., *Eur. J. Immun.*, **4**, 701 (1974).
- Tomasi, T. B., and Zigelbaum, S. D., *J. clin. Invest.*, **42**, 1552 (1963).
- Buckley, R. H., and Dees, S. C., *New Engl. J. Med.*, **281**, 465 (1969).
- Kaufman, H. S., and Hobbs, J. R., *Lancet*, **ii**, 61 (1970).
- Taylor, B., et al., *Lancet*, **ii**, 111 (1973).
- Eisen, H. N., Simms, E. S., and Potter, M., *Biochemistry*, **7**, 4126 (1968).
- Comoglio, P. M., and Guglielmo, R., *Immunology*, **25**, 71 (1972).
- Porter, R. R., *Meth. Med. Res.*, **3**, 256 (1950).
- Hunter, W. M., and Greenwood, F. C., *Nature*, **194**, 495 (1962).
- Stokes, C. R., Taylor, B. W., and Turner, M. W., *Lancet*, **ii**, 485 (1974).
- Walker, W. A., Isselbacher, K. J., and Bloch, K. J., *J. Immun.*, **111**, 221 (1973).
- Heremans, J. F., and Heremans, M. Th., *Acta med. scand., Suppl.*, **367**, 27 (1961).
- Brown, W. R., Newcomb, R. W., and Ishizaka, K., *J. clin. Invest.*, **49**, 1374 (1970).
- Bienenstock, J., *Progress in Immunology II*, 4 (edit. by Brent, L., and Holborow, J.), 197 (North Holland, Amsterdam, 1974).
- Hill, I. R., and Porter, P., *Immunology*, **26**, 1239 (1974).

Erratum

In the article "Role of macrophages in *in vitro* induction of T-helper cells" by P. Erb and M. Feldmann (*Nature*, **254**, 352; 1975), page 353, column 2, line 3, for presence read absence.

In the article "Centrometric asymmetry and induction of translocations and sister chromatid exchanges in mouse chromosomes" by M. S. Lin and R. L. Davidson (*Nature*, **254**, 354; 1975) Figs 1 and 3 were transposed. The captions are correct as they stand.

In the article "Synthesis of ribosomal RNA during sporulation in *Bacillus subtilis*" by D. Testa and R. Rudner (*Nature*, **254**, 632; 1975) Fig. 1 was rotated through 90°. The time scale should have been on the abscissa.

In the article "Crystallisation of troponin-C" by D. Mercola, B. Bullard and J. Priest (*Nature*, **254**, 634; 1975) the results in the first sentence of paragraph 4 are not attributable to ref. 12.

reviews

THE distinguished biochemist Parnas once invited me to look at his collection of offprints. At first there seemed to be nothing unusual about them; all I saw were the familiar rows of labelled boxes. Then Parnas explained what was unusual: the offprints, from hundreds of authors, were catalogued under the names of only five or six biochemists. "When I read a paper", Parnas said, "I recognise in it the ideas of one or other of no more than six creative men—Hopkins, Englehardt . . .—and I classify the paper accordingly".

*The Creative Process in Science and Medicine** quotes a remark by Lewis and Randall (from a treatise on thermodynamics) which is similarly perceptive. The edifice of science is like that of a cathedral built by a few architects and numerous workmen. A scientist does not have to be creative in order to be a valuable member of the scientific community. But the grand design of the edifice of science depends on the creative activities of a mere handful of workers.

What is the secret of this rare creative faculty? In May 1974 C. H. Boehringer Sohn organised a symposium to ask this question. Twenty four people assembled at Kronberg for a couple of days to converse about the creative process in science and medicine. This book records their conversations.

Most unscripted discussions on radio and television are vapid and frothy: they rarely rise to a level which makes it worth while to set them in print. This little book demonstrates that unscripted discussions can be illuminating as well as lively. For two reasons, these conversations are well worth printing: first, some of the contributors are in the front rank of creative scientists (they include four Nobel prizewinners); second, the conversations have been edited with great skill, so that they cleverly combine informality with clarity.

Monod opened the discussions by giving his views about what he called "ingredients" of the creative process,

illustrated by the steps of reasoning which went into some of his own classical work on enzyme action. The theme was taken up by Krebs, Popper, Eisenberg, Eigen, Eccles, Tinbergen, and others. Occasionally the distinguished party strayed from the theme or followed some wayward idea like kittens playing with a ball of wool; but in the end a remarkable consensus

Playing with ideas

Eric Ashby



emerged about the conditions under which the creative process operates.

There are certain ground rules. One should not, for example, entertain any hypothesis unless it is one which "can be falsified even by an imaginary experiment". One needs to have "technical courage"—never be frightened of breaking a rule or switching to a new technique. Orthodoxy, says one speaker, is the enemy of creativity. One has to have the "capacity to pay attention to oddities"; a flair for elegance of thought, a sense of symmetry; something the symposium summed up as "good taste".

There are, too, certain environmental prerequisites. "You cannot be creative",

says Eigen, "until you have new experiences". Eccles is even more specific. The experiences have got to be challenging: "Problems with ideas that are in conflict are an important challenge. Conflict is one of the most essential conditions". There is agreement about the necessity for an incubation period—familiar to those engaged on even the most modest kinds of creative work—when the ideas are put out of mind for a time and (sometimes) become illuminated in a fresh and exciting way when they are re-examined. 'Playing with ideas' is an important element in the creative process, and there is some interesting talk as to whether the scientist has something to learn from the way children and animals engage in play.

What is the neurological and anatomical basis for creative thought? Eccles gives a lucid summary of the intricate way in which intentions in the brain are translated into action by muscles. But since uncreative people and even higher animals have similarly intricate neurological 'outfits', this gives no clue to the physical basis of the creative process. Indeed, one of the refreshing impressions one gets from reading this record of the conversations is of the readiness of creative scientists to admit that in the study of human behaviour there is what Popper calls "*Erdenrest*", "a remainder or residue which is not rational". This is not to say that rational thought must not be pushed to the very limits of human capacity; but it does mean that one must not exclude the possibility that some phenomena lie beyond rational thought, and will remain out of reach whatever advances science makes. Perhaps a better way to put this would be to say that if some human achievements are explained rationally, the achievements themselves vanish. Einstein is reported to have made a remark which illustrates this. Asked whether everything could ultimately be expressed in scientific terms he replied: "Yes, that is conceivable, but it would make no sense. It would be as if one were to reproduce Beethoven's Ninth Symphony in the form of an air pressure curve".

These conversations will not serve as much of a guide to aspirants hoping to acquire creativity. But they will help the ordinary mortal to appreciate more fully how the creative process works in those rare persons lucky enough to have the capacity for it. □

**The Creative Process in Science and Medicine*. (Proceedings of the C. H. Boehringer Sohn Symposium, May 1974.) Edited by Hans A. Krebs and Julian H. Shelley. Pp. xii+138. (Excerpta Medica: Amsterdam; American Elsevier: New York, 1974.) Dfl.52; \$21.75.

Solid state and orbital theories

Solid State and Molecular Theory: A Scientific Biography. By John C. Slater. Pp. 357. (Wiley-Interscience: New York and London, March 1975.) £10.25.

JOHN SLATER started research in 1923 and was from the first concerned with atomic, molecular and solid state theory. Nobody could be better qualified to tell the story of the development of these subjects, and this Slater has done in a very personal way.

Of the days before quantum mechanics there are some curious sidelights. On a visit to the Cavendish and Copenhagen he became convinced of the existence of photons, but found that Bohr refused to consider the idea and dissuaded him from publishing—and that still rankles. Then came 1925 and 1926 and the startlingly sudden formulation of quantum and wave mechanics, which Slater describes. Of this period Slater says there seemed to be among theoretical physicists two types: one prosaic, matter of fact and willing to indicate the argument behind what he did; the other like a magician, waving his hands as if he were drawing a rabbit out of a hat. Though admiring—

who could not?—the achievements of some of the latter, Slater puts himself in the former category. I would agree, and it is in thorough and detailed work on solids and molecules, a description of which forms the main part of the book, that he has made his greatest contributions. Particularly charming is his account of the early days of many-electron wave functions, when Wigner, Hund and others entered the field with their "*Gruppenpest*", the pest of group theory, as disgruntled people who had never studied the subject in school described it. By his introduction of the "Slater determinant" he provoked the remark that "Slater has slain the *Gruppenpest*"—and, as he says, his paper was universally popular.

The account of the application of advanced computers to problems of molecular wave functions occupies the last part of the book. Slater has been involved with this for the last 10 years since he retired from MIT. As he points out, this kind of work had to await the development of the computer, and could not have been done earlier. In this Slater is clearly in his element. His likes and dislikes come out strongly, both as regards persons and methods. He likes a clearly defined problem, complicated enough to be difficult but simple enough for the computer of the day. Approximate methods, valid for even more complicated problems, are less to his taste. Thus the pseudopotential is not mentioned and the work of the English school using such methods is rather summarily dismissed. Curiously, for a physicist who has done so much for metal theory, there is no mention of Landau's work on the nature of the Fermi surface. Slater, one feels, draws a line round himself and the computer, and those outside it are the handwaving magicians. But within this circle Slater's achievements are second to none and all his friends and colleagues will hope that his present very successful work will continue for many years. **Nevill Mott**

Orbital Theories of Molecules and Solids. Edited by N. H. March. Pp. xiv + 385. (Clarendon: Oxford; Oxford University Press: London; November 1974.) £10.75.

IN 1973 Charles Coulson celebrated his 60th birthday and part of the tribute paid to him by his students and close collaborators is this book. It is essentially a review of the current state of those research fields to which the greater part of his scientific life was dedicated.

The book divides into two distinct areas in spite of all the efforts of the authors to maintain continuity. The solid state part begins with a general

survey by Pincherle of the present state of band theory calculations. Then Altmann presents a detailed review of the often misunderstood cellular methods and shows in a convincing way that their neglect is not justified. If those papers stress the orbital concept in its delocalised form the next two redress the balance by considering the atoms. The editor, March, contributes a masterly review of the theory of the potential around atoms and around defects in crystals and shows how many-body effects can be assimilated into what may be considered a typical orbital concept. The different kinds of defect that can occur in crystals of different types is the subject of Lidiard's chapter. He demonstrates, more convincingly than in any other chapter, the fruitful interplay between molecular and solid state ideas. The extent to which this field has been organised and analysed theoretically is impressive.

The molecular half of the book begins with a most useful account by McWeeny of the electronic properties of molecules. He stresses the role of density matrices in unifying and simplifying these properties and although he refers to a large number of spin and magnetic properties the reader is given simple interpretations of their origin. Although Coulson is best known for his molecular orbital work he was prepared to use other theories when they suited his purpose. It is fitting, therefore, that the next chapter, by Balfint-Kurti and Karplus on atoms in molecules, should use valence bond ideas to obtain calculated molecular energies in better accord with experiments than simple molecular orbital results could ever give.

The book concludes with two articles which carry the subject into different regions. Murrell presents a careful look at the theory of intermolecular forces and shows that, though caution is needed in some instances, the traditional division into additive components can still be justified despite all the complications of a more sophisticated theory. Craig brings the book back to the theme of molecules and solids with an up-to-the-moment account of the theory of excitations in molecular solids. Here, the nature of the free molecule and of its association in the solid, a major interest of Coulson's, are seen to contribute equally to an understanding of the complicated structure of the excited states of molecules in crystals.

The death of Charles Coulson came as a bitter blow to those who looked to him for new research ideas and new insights into old truths. This book which demonstrates both the enduring quality of his own basic ideas and the sound training he gave his students will be a worthy memorial to him. **G. G. Hall**

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Cells in animals . . .

Differentiation and Growth of Cells in Vertebrate Tissues. Edited by G. Goldspink. xi+323 (Chapman and Hall: London; distributed in the USA by Halsted Press; December 1974.) £10.30.

As with most multi-author works, this book is largely a heterogeneous collection of the interests of the contributors. The dustcover states that the aim of the book is to "bring to the fore a relatively neglected part of developmental biology . . . namely the development of the cells in different tissues of the body". This the book achieves for seven selected tissues, but only at the expense of continuity and by becoming repetitious. The first and last articles are, however, exceptions in that they are not restricted to any type of cell.

The book is divided into nine chapters, seven of which deal separately with the growth and development of nerve cells, muscle, bone and connective tissue, skin, gonads, blood cells and lymphoid tissue, in that order. It opens with an excellent account by Jane E. Brown and K. W. Jones of the current concepts of differentiation, which effectively explains well established phenomena of differentiative processes in terms of molecular biology. There is an up-to-date discussion of topics like gene activation, transcriptional control and biogenesis of messenger RNA, but one cannot help wondering how long it will be before some of the ideas become outdated, considering the rapid changes taking place in this field.

The second chapter is a very brief account by M. Jacobson of how nerve cells grow and differentiate, much of it dealing with positional specificity of connections in the visual system. The editor's contribution is a well balanced blend on the morphology of developing muscle cells and the control of biosynthesis of muscle proteins. J. J. Pritchard's description of bone and connective tissue is largely at the histological level and so is that of skin by F. J. Ebling, except that the latter takes an evolutionary or comparative approach to development which makes his chapter particularly interesting. Descriptive morphology (at the ultrastructural level) is also the theme of B. Gondos' article on gonads. P. F. Harris gives a comprehensive survey of the origin and maturation of all the major classes of red and white blood cells, with a good selection of electron micrographs. Margaret Manning and J. D. Horton lay more emphasis on function than on morphogenesis in discussing the role of lymphoid cells in immunological defence.

The book ends rather abruptly with D. Bellamy's cursory account of the different theories on ageing and cell death. A discussion of senescence and death in a book on developmental biology is undoubtedly most desirable and it is

indeed a pity that in this case it is so superficial. Some of the theories of ageing are complicated or outdated and the brevity makes it especially difficult to sort out firm experimental evidence from vague hypotheses.

This book will be particularly valuable for the postgraduate or postdoctoral research worker who is not yet a specialist in any of the fields covered in the book. It is well presented with a summary and useful bibliography at the end of each chapter. The pleasant print and good photographic reproduction are marred by the fact that about 30 pages of one chapter are upside down—perhaps my copy was an odd one. **J. R. Tata**

. . . and plants

The Physical Biology of Plant Cell Walls. By R. D. Preston. Pp. xiv+491. (Chapman and Hall: London; distributed in the USA by Halsted Press; September 1974.) £12.00.

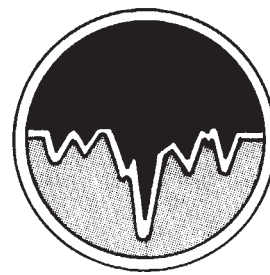
THE plant cell wall has been examined using numerous physical, chemical and biochemical techniques. This book concentrates on the results produced by physicochemical analyses. These methods, such as X-ray diffraction, electron microscopy, and the measurement of mechanical properties, show clearly the properties and structure of the microfibrils in the wall but do not indicate much about the matrix materials. Thus, this text is mainly about cellulose and represents a comprehensive and authoritative account of the deposition, organisation, structure and possible mechanisms of the biosynthesis of that important structural polysaccharide.

As some of the physical methods used may not be familiar to botanists the author develops very clearly the elementary theory of the techniques and the practical difficulties of their application. Each method is discussed, so that the limitations and the pitfalls in the interpretations of the data obtained from the technique can be clearly appreciated. The results are presented as a logical sequence and are critically appraised to show how the ideas and concepts of microfibrillar structure and organisation have developed.

Although the author discusses various conflicting viewpoints and describes the importance and the significance of the different theories, it is usually very clear to which theory the author himself subscribes. But he takes no stand without giving a thorough and detailed argument in its favour.

The book is well illustrated with clear diagrams and numerous electron micrographs. It is well indexed and easy to read. Altogether it represents a very useful and even essential addition to any library connected with a laboratory dealing with plant material and tissues.

D. H. Northcote



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Edited by P.J. ROLLS

Hardback: June 1975: 652 pages: tone and line illustrations: 412 12330 4: £15.00

This book is a complete collection of over 100 papers given at the Eleventh International Congress. The book will be of interest to research workers in optics, photography, lasers, optical engineering and related fields.

Bacteriophages

J. DOUGLAS

Hardback: July 1975: about 144 pages: illustrated: 412 12630 3: £5.00

Science Paperback: 412 12640 0: £2.50

Written in a clear straightforward style, with many illustrations, this book is a concise but comprehensive introduction to bacteriophage biology. It covers structure and function, 'growth', survival and death. The contribution of bacteriophages to fundamental biological research and their role in medicine and industry are examined. The wide range of microbiological techniques which have led to the discovery of many of the precepts of both bacteriophage and general biology are also discussed.

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announcements

Awards

The Institution of Mining and Metallurgy has awarded its **Gold Medal** to **J. Lumsden** for contributions to physical chemistry of extractive metallurgy, particularly the thermodynamics of smelting operations.

The Linnean Society has awarded **Gold Medals** to **P. M. Sheppard, FRS**, and **A. S. Watt, FRS**. The **H. H. Bloomer Award and Medal** (for a distinguished amateur naturalist) goes to **E. S. Edees**, author of *Flora of Staffordshire*.

The National Research Council (Washington, DC) has presented **H. Isbell** with the **Nathan B. Eddy Memorial Award** for research into the problems of drug dependence.

The trustees of the Lady Tata Memorial Trust have made awards to the following: **C. Guibout, J. Phillips, L. Vardimon** and **M-C. Groessens Van-Dyck**.

Appointment

The University of Newcastle upon Tyne has appointed **E. S. Page** and **J. R. O'Callaghan** as pro-vice-chancellors.

Miscellaneous

Chemistry award. The Meldola Medal and Prize (of £100) will be made in 1976 to a British chemist (under age 30) who shows the most promise as indicated by his or her published work brought to the notice of the Royal Institute of Chemistry before December 31, 1975. Further information from: The President, Royal Institute of Chemistry, 30 Russell Square, London WC1B 5DT, UK.

Beilby medal and prize. This award will be made during 1976 to a British scientist (under age 40) in recognition of independent original work of exceptional merit, carried out over a period of years and involving the development and application of scientific principles in chemical engineering, fuel technology or metallurgy, in their modern interpretations. Closing date: December 31, 1975. Further details from: The Convener of the Administrators, Sir George Beilby Memorial Fund, Royal Institute of Chemistry, 30 Russell Square, London WC1B 5DT, UK.

Person to Person

Barnacle research. Graduate student compiling a bibliography of the genus *Balanus* would like to include thesis and dissertation contributions; if any such work known concerning *Balanus* in any way, please send postcard including author, degree, title, year, institution and short summary if possible (M. Landau, New York Ocean Science Laboratory, Montauk, New York 11954).

Astronomical catalogues. Bibliography of catalogues for the period 1951-75 almost completed. Any information, especially concerning obscure or unpublished work, gratefully received (Mike Collins, Senior Information Scientist, INSPEC, Station House, Nightingale Road, Hitchin, Hertfordshire SG5 1RJ, UK).

Tornadoes and vortices. Scientist researching into the mechanism of funnel clouds wishes to see original film sequences showing tornadoes and other vortices. Could arrange to visit owner's premises to make videotape recordings of suitable material (J. M. Heighes, JOMAR, College Road, College Town, Sandhurst, Berkshire, UK).

New York-London. Wanted September 1, 1975-July 15, 1976. Two bedroom flat convenient for King's College (Strand) to rent or exchange for same on East Side of Manhattan, within walking distance of Rockefeller University, Cornell-New York Hospital, Sloan-Kettering; available immediately (Professor A. Haschemeyer, Hunter College Box 741, New York, New York 10021).

London accommodation. Canadian family of four urgently require accommodation in London area from August 1, 1975 for six months (Dr R. J. Riopelle, Department of Zoology, Room B6, University College, London WC1E 6BT, UK).

There will be no charge for this service. Send items (not more than 60 words) to Robert Vickers at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

International meetings

August 14-20, **Low temperature physics**, Otaniemi, Finland (LT 14, c/o Low Temperature Laboratory, Helsinki University of Technology, SF-02150, Otaniemi, Finland).

August 15-29, **Cosmic rays**, Munich (K. Pinkau, Max-Planck-Institute for Extraterrestrial Physics, D-8046 Garching/Munich, Federal Republic of Germany).

August 16-23, **Plant pathology**, Munich (Congress Plant Pathology, Biologische Bundesanstalt, Messeweg 11/13, D-3300 Braunschweig, Federal Republic of Germany).

August 17-22, **Biomedical engineering**, Edinburgh (The Secretariat, Biological Engineering Society Conference, Princess Margaret Rose Orthopaedic Hospital, Edinburgh EH10 7ED, UK).

August 17-23, **Biodegradation**, Kingston, Rhode Island (Dr R. W. Traxler, Chairman, 231 Woodward Hall, University of Rhode Island, Kingston, Rhode Island, 02881).

August 17-25, **Long-term climatic fluctuations**, Norwich, UK (Conference organiser, World Meteorological Organisation, PO Box 5, 1211 Geneva 20, Switzerland).

August 18-20, **Nitrogen as a water pollutant**, Copenhagen (J. Lonholdt, Department of Sanitary Engineering, Technical University of Denmark, Lyngby, Denmark).

August 18-22, **Phenomena in ionised gases**, Eindhoven, The Netherlands (F. J. de Hoog, Eindhoven University of Technology, PO Box 513, Eindhoven, The Netherlands).

August 18-30, **Pacific science**, Vancouver (13th Pacific Science Congress 1975, University of British Columbia, Vancouver 8, Canada).

August 19-22, **Cyclotrons and their applications**, Zurich (Mr W. Joho, Swiss Institute for Nuclear Research, 5234 Villigen, Switzerland).

August 20-22, **High polymers**, Hamilton, Ontario (J. Prud'homme, Secretary-Treasurer, 18th Canadian High Polymer Forum, Department of Chemistry, University of Montreal, PO Box 6210, Montreal, Quebec, Canada).